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Fungal Catabolism of Crown Gall Opines

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This study was conducted to determine the capacities of 37 fungi to utilize various crown gall opines as their sole carbon and nitrogen source. One strain of *Fusarium solani*, two of *Cylindrocarpon destructans*, and six of *Cylindrocarpon heteronema* catabolized octopine, mannopine, octopinic acid, succinamopine, or a combination of these opines. One *C. heteronema* and one *Fusarium dimerum* strain grew only on succinamopine. None of the fungal isolates had the ability to grow on nopaline. The catabolism of opines by fungi was confirmed by the disappearance of the opine from the growth medium and by an increase in final mycelial dry weight with rising initial concentration of test substrate. This study thus shows that the catabolism of opines is not restricted to bacteria.

Virulent strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* induce crown gall and hairy root disease, respectively. The introduction of part of their Ti or Ri plasmid T-DNA into the plant genome induces the synthesis of plant hormones and novel metabolites, termed opines (15).

Opines are classified as phosphorylated sugars, imino diacids, or mannityl opines (6, 22). Opines from the two latter groups contain an amino acid moiety, referred to as the parent amino acid. The imino diacid group is further subdivided into the pyruvate and 2-ketoglutarate subgroups. Pyruvate-derived opines have a D-Ala–L-amino acid stereochemistry, while most 2-ketoglutarate-derived opines have a D-Glu–L-amino acid stereochemistry, and a few have an L-Glu–L-amino acid stereochemistry.

The opines are released into the vicinity of the plant (17, 18) and may induce the expression of catabolic genes in *Agrobacterium* strains (22). The degradation of nopaline, octopine, and octopinic acid requires the activity of at least an opine permease and an opine oxidase. These opines are broken down into 2-ketoglutarate and arginine (21), pyruvate and arginine (16), and pyruvate and ornithine (13), respectively. The metabolic pathways of mannityl opine catabolism are not completely understood. They involve at least two transport systems, one induced by mannopinic acid or agropinic acid and the other induced by mannopine or agropine (11). When mannopinic acid induces its specific transport system, this molecule is cleaved into mannose and glutamate, and then mannose is isomerized to fructose (11). Mannopine may be converted to agropine by lactonization (10), but the complete degradative scheme of this molecule is not understood. Little is known about the metabolic steps involved in the utilization of succinamopine, but they probably include activity by a dehydrogenase which is responsible for the cleavage of this opine (6).

To date, opine catabolism has been observed in *Agrobacterium* strains and in some strains of coryneform bacteria (23), *Pseudomonas* spp. (1, 2, 20), and a *Rhizobium* sp. (22). Moreover, it was recently observed that three strains of *Cylindrocarpon heteronema* and one of *Fusarium dimerum* isolated from a pear crown gall were able to catabolize opines (P. Dion, C. S. Nautiyal, C. Beauchamp, and W. S. Chilton, J. Cell Biochem. Supp. **13A**:167, 1989). Consequently, the aim of the present study was to evaluate the ability of fungi to use opines as their sole source of carbon, nitrogen, and energy. The disappearance of opines and their parent amino acids from the growth medium was monitored, and the accompanying mycelial growth was measured.

(A preliminary account of part of this work was presented at the 39th annual meeting of the Canadian Society of Microbiologists, Laval, Quebec, Canada, June 1989.)

MATERIALS AND METHODS

Fungal strains and inoculum preparation. The strains or isolates used in this study are described in Table 1. Of these, three strains of *C. heteronema* (AR-12, AR-13, and AR-22) and one of *F. dimerum* (AR-14) were isolated from a pear crown gall on opine-containing medium (C. S. Nautiyal and P. Dion, unpublished data). The other fungi were obtained on media not containing opines. Two types of inoculum were used in the growth assays. In the first series of experiments, fungi were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) at room temperature for 7 days. Mycelial plugs (4 mm) were taken from the edge of the colony, and the agar was aseptically trimmed to a depth of 1 mm. Plugs were then added to test tubes filled with 2 ml of opine-containing medium. Fungi which gave a positive response in this first test were further evaluated as follows. Cultures were grown on potato dextrose agar for 14 days at room temperature. Fungal spores were harvested with 10 ml of sterile distilled water and diluted to a density of 10^7 spores · ml^{-1}. Each test tube, containing 10 ml of the test compound medium, was then inoculated with 0.1 ml of this spore suspension.

Media and growth conditions. The AT salts of Guyon et al. (14) supplemented with biotin (0.1 mg · $liter^{-1}$), thiamine (0.1 mg · $liter^{-1}$), nicotinic acid (1 mg · $liter^{-1}$), and pantothenic acid (1 mg · $liter^{-1}$) were used as the basal medium (AT-B). The opines tested were added to AT-B at a final concentration of 0.8 g · $liter^{-1}$, except for a 50:50 mixture of DL- and LL-succinamopine, which was used at 1.6 g · $liter^{-1}$. The AT-glucose-nitrogen medium (AT-GN) consisted of AT-B with glucose (2 g · $liter^{-1}$) and ammonium sulfate (1 g · $liter^{-1}$). Carbon, nitrogen, and vitamin sources were

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TABLE 1. Fungi used in this study

Fungal species	Strain	Origin	Source[a]
Alternaria sp.	86-20	*Solanum tuberosum*	P. Thibodeau
A. solani	241	*S. tuberosum*	C. Richard
Botrytis sp.	86-21	*S. tuberosum*	P. Thibodeau
Colletotrichum coccodes	32	*S. tuberosum*	C. Richard
Cylindrocarpon destructans	88-01	Unknown	A. Caesar
	DAOM-185722	*Pyrus* sp.	BRI
	4895	*Vitis* sp.	UAMH
C. didymum	DAOM-155445	*Poa pratensis*	BRI
	4888	*Pyrus* sp.	UAMH
C. heteronema	AR-12, AR-13, AR-22	*Pyrus* sp.	P. Dion
	88-02, 88-03	Unknown	A. Caesar
	31	Soil	C. Richard
	789, 790, 816	*Fagus grandifolia*	R. Cochon
	48896	*Swietenia mahogani*	ATCC
Fusarium coeruleum	1620	*Solanum tuberosum*	R. Hall
F. dimerum	16553	Poultry feed	ATCC
	3315	Unknown	UAMH
	DAOM-149431	*Brassica oleracea*	BRI
	DAOM-170311	*Ruscus* sp.	BRI
	RM-1446, RM-3017	Soil	R. A. A. Morrall
	AR-14	*Pyrus* sp.	P. Dion
F. oxysporum	304	*Solanum tuberosum*	C. Richard
F. sambucinum	86-26	*S. tuberosum*	A. Murphy
F. solani	86-25	*S. tuberosum*	A. Murphy
Phoma exigua	309	*S. tuberosum*	C. Richard
Rhizoctonia solani	15B	*Nicotiana tabacum*	D. A. Brown
Thielaviopsis basicola	1	*N. tabacum*	D. A. Brown
Verticillium dahliae	175	*S. tuberosum*	C. Richard
	S. tube 8.169	*S. tuberosum*	L. Tartier
V. albo-atrum	SBA-2, SBA-4	*S. tuberosum*	L. Tartier

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adjusted to pH 7 and filter sterilized through 0.2-μm-pore-size membrane filters (Millipore Ltd., Mississauga, Ontario, Canada) before being added to the medium. Fungi introduced as mycelial plugs were tested in three replicates. The results in AT-B and AT-GN were used as standards for negative and positive responses, respectively. Growth was monitored for up to 28 days of incubation at 25 ± 2°C with shaking at 50 rpm (model G10; New Brunswick Scientific Co., Inc., Edison, N.J.). In a second set of experiments, the opines and their corresponding parent amino acids were tested at 0, 1, 2, 3, and 4 mM. The inocula consisted of fungal spores, and the incubation conditions were similar to those described above. Three replicates were used for each combination of substrate and concentration. After 20 days of growth, the mycelia were harvested, dried at 50 ± 5°C for 24 h, and weighed.

Utilization of opines and amino acids. The disappearance of opines from the culture medium was monitored weekly for the first series of experiments. In the second series, the disappearance of opines and amino acids was evaluated every other day. Twenty-microliter samples of all amino acids and opines, except succinamopine, for which 40-μl samples were used, were spotted on 3MM paper (Whatman, Inc., Clifton, N.J.). Papers were subjected to high-voltage electrophoresis (ca. 40 V/cm) in pH 9.8 buffer for mannopine (19), pH 2.8 buffer for succinamopine (8), or pH 1.8 buffer for all the other compounds (19). Papers were then stained with silver nitrate for mannopine (24), silver nitrate-mannitol for succinamopine (8), ninhydrin to detect octopinic acid and all the amino acids (9), and phenanthrenequinone for octopine (25).

Chemicals. Octopine, octopinic acid, mannopine, nopaline, and amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo.). The 50:50 mixture of DL-succinamopine and LL-succinamopine was synthesized as previously described (7).

RESULTS

Utilization of opines by fungi. Vigorous mycelial growth was recorded for all tested fungi in AT-GN, but poor to almost no growth was observed in AT-B. None of the *Alternaria* spp., *Botrytis* sp., *Colletotrichum* sp., *Phoma* sp., *Rhizoctonia* sp., *Thielaviopsis* sp., or *Verticillium* spp. were able to catabolize a crown gall opine. However, 9 of 12 *Cylindrocarpon* isolates tested exhibited the ability to grow on some of the test opines. Similarly, 2 of 10 *Fusarium* isolates were opine utilizers. The positive fungi utilized up to four different opines (Table 2). None of the fungi tested grew on nopaline. The disappearance of opines from the culture supernatant was correlated with visible fungal growth.

Characteristics of opine and amino acid utilization. Mannopine disappeared from the culture medium in 4 to 8 days, whereas glutamine completely disappeared in 4 to 6 days (Table 2). Two other silver-reducing substances became detectable during cultivation of the fungi in the medium

TABLE 2. Number of days required for the complete catabolism of opines and amino acids at 4 mM by *Cylindrocarpon* and *Fusarium* species

Fungal species and strain	No. of days required for complete catabolism of[a]:								
	MOP	GLN	OCT	ARG	OCA	ORN	L-SAP	D-SAP	ASN
Cylindrocarpon destructans									
88-01	—	ND	8	6	8	6	6	8	4
4895	—	ND	10	6	—	ND	—	P	ND
Cylindrocarpon heteronema									
AR-12	8	4	2	4	—	ND	4	P	2
AR-13	6	6	2	4	—	ND	4	P	2
AR-22	8	4	2	4	—	ND	6	P	2
88-02	—	ND	4	4	14	8	4	P	4
88-03	—	ND	12	6	14	6	6	P	4
31	4	4	2	4	—	ND	—	P	ND
790	—	ND	—	ND	—	ND	16	P	4
Fusarium dimerum									
AR-14	—	ND	—	ND	—	ND	4	P	4
Fusarium solani									
86-25	4	4	2	4	—	ND	—	P	ND

[a] Abbreviations: MOP, mannopine; GLN, glutamine; OCT, octopine; ARG, arginine; OCA, octopinic acid; ORN, ornithine; L-SAP, LL-succinamopine; D-SAP, DL-succinamopine; ASN, asparagine; —, no growth; ND, not determined; P, present throughout 20 days of observation.

containing mannopine. These compounds comigrated with agropinic acid and mannopinic acid standards and were designated as such in the present work. In the culture supernatant of strain AR-12, mannopine and agropinic acid were still detected after 6 days of growth, but both had disappeared after 8 days. In addition, mannopinic acid was detected only on day 8 of growth. For strain AR-13, mannopine was present in the incubation medium for 4 days and then disappeared. Its disappearance was concomitant with the appearance of mannopinic acid, which disappeared from the culture supernatant after an additional 6 days. For strain AR-22, mannopine was present for 6 days, agropinic acid was present throughout the 20 days of experimentation, and mannopinic acid was detected, in small amounts, between days 6 and 20 of growth. For strain 88-31, mannopine was used within 4 days, at which time agropinic acid appeared. This opine was no longer detected after 6 days of growth, and mannopinic acid was never recorded. For strain 86-25, mannopine disappeared from the culture supernatant after 4 days of growth, while agropinic acid and mannopinic acid were never detected. Culture supernatants of strains unable to catabolize mannopine accumulated agropinic acid, but not mannopinic acid, over a 20-day period.

All strains capable of utilizing octopine completely degraded this opine within 2 to 12 days and completed the catabolism of its parent amino acid, arginine, within 4 to 6

TABLE 3. Effects of opines and amino acids on mycelial dry weight of *Cylindrocarpon* and *Fusarium* species after 20 days of incubation at 25°C

Fungal species and strain	Mycelial dry wt ($\mu g \cdot ml^{-1}$) following growth on[a]:							
	MOP	GLN	OCT	ARG	OCA	ORN	SAP	ASN
Cylindrocarpon destructans								
88-01	ND	ND	393 ± 50*	227 ± 12*	377 ± 52*	330 ± 62*	247 ± 20*	225 ± 125*
4895	ND	ND	307 ± 03	185 ± 05	ND	ND	ND	ND
Cylindrocarpon heteronema								
AR-12	343 ± 18*	153 ± 23*	300 ± 50*	220 ± 20*	ND	ND	140 ± 45*	77 ± 27*
AR-13	273 ± 107*	160 ± 20	287 ± 19*	133 ± 33*	ND	ND	120 ± 6	95 ± 5
AR-22	393 ± 20*	173 ± 20*	327 ± 37*	127 ± 23*	ND	ND	140 ± 20*	120 ± 60
88-02	ND	ND	247 ± 44*	160 ± 57*	293 ± 45*	160 ± 68*	110 ± 35	77 ± 32
88-03	ND	ND	233 ± 28*	163 ± 55*	320 ± 25*	207 ± 32*	183 ± 18*	93 ± 26
31	333 ± 71*	117 ± 18*	310 ± 25*	203 ± 38*	ND	ND	ND	ND
790	ND	ND	ND	ND	ND	ND	150 ± 35*	57 ± 29*
Fusarium dimerum								
AR-14	ND	ND	ND	ND	ND	ND	115 ± 5	30 ± 15
Fusarium solani								
86-25	333 ± 30*	150 ± 42*	273 ± 27*	180 ± 40*	ND	ND	ND	ND

[a] Initial concentrations of test substrates ranged from 0 to 4 mM. The data shown are for final mycelial dry weight on an initial substrate concentration of 4 mM. These results are expressed as the means of three replicates ± standard errors. For each strain-test substrate combination, an asterisk indicates a significant increase in mycelial dry weight in the presence of rising initial concentrations of the substrate, as shown by regression analysis. Abbreviations: MOP, mannopine; GLN, glutamine; OCT, octopine; ARG, arginine; OCA, octopinic acid; ORN, ornithine; SAP, succinamopine; ASN, asparagine; ND, not determined.

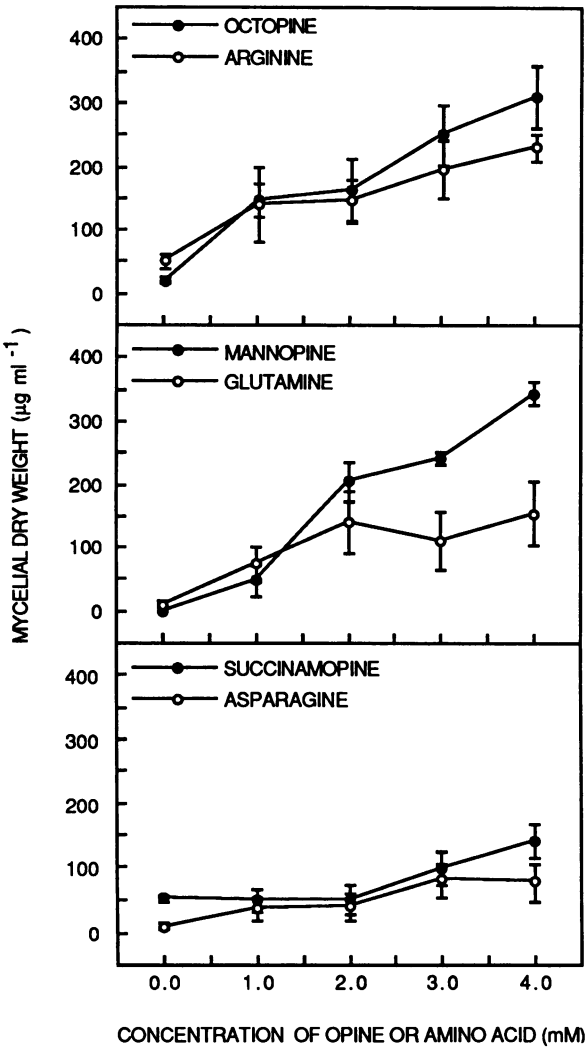

FIG. 1. Growth response of *C. heteronema* AR-12 in AT salts supplemented with increasing concentrations of opines or amino acids. Growth was measured after 20 days. Means of three replicates ± standard errors.

days (Table 2). Octopine degradation by strains 88-01 and 4895 was accompanied by the transient accumulation of arginine in the culture supernatant at day 2; by day 4, this intermediate of octopine catabolism had disappeared. The catabolism of octopinic acid and succinamopine was slower than that of the corresponding parent amino acids. LL-Succinamopine was utilized by eight strains, while DL-succinamopine was utilized only by strain 88-01, the only strain capable of using both isomers of succinamopine.

After it was established that some fungi were capable of degrading various opines, those fungi were evaluated for their ability to utilize opines for the production of mycelium from a spore inoculum. Following 20 days of incubation in the absence of an added carbon and nitrogen source, the mycelial dry weights averaged only 42 ± 29 $\mu g \cdot ml^{-1}$ (results not shown). Most fungal isolates showed significant increases in mycelial dry weight in the presence of rising initial concentrations of opines and parent amino acids (Table 3; Fig. 1 and 2). One exception was the slight growth response to succinamopine and asparagine exhibited by many strains. Only strain 88-01 (Fig. 2) showed a growth

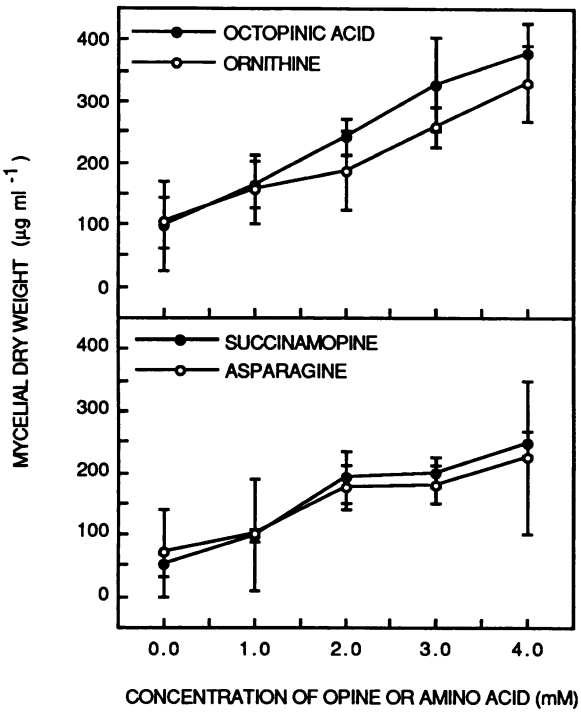

FIG. 2. Growth response of *C. destructans* 88-01 in AT salts supplemented with increasing concentrations of opines or amino acids. Growth was measured after 20 days. Means of three replicates ± standard errors.

response to succinamopine and asparagine which was similar to that obtained with the other opines and amino acids. The final mycelial dry weight was lower when growth had occurred on the parent amino acid instead of the corresponding opine (Table 3; Fig. 1 and 2).

DISCUSSION

Representatives of nine genera of phytopathogenic fungi were evaluated for the utilization of opines as their sole carbon, nitrogen, and energy source. Positive isolates were found in only two of these genera. The disappearance of mannopine, octopine, octopinic acid, or succinamopine from the growth medium was concomitant with an increase in mycelial dry weight; consequently, these compounds were taken up from the medium, catabolized, and used to support fungal growth. In contrast, nopaline was not catabolized by these fungi.

Final mycelial dry weight increased with an increase in initial concentration of opine or parent amino acid. For a given initial concentration of test substrate, final mycelial dry weights were generally higher with opines than with the corresponding amino acids. This difference was expected, because opines contain tricarboxylic acid or sugar moieties conjugated to the amino acid moiety, providing extra carbon. The aim of this study was not to define conditions for optimal incorporation of the provided substrates into cellular material. In particular, in some cultures the C/N ratio may have become limiting for growth during the course of incubation. On average, the economic coefficients (mycelial dry weight divided by the weight of carbon and nitrogen consumed) were respectively 27 and 24% for mannopine and glutamine, 30 and 21% for octopine and arginine, 40 and 34%

for octopinic acid and ornithine, and 14% for both succinamopine and asparagine. These ratios show that a significant proportion of the carbon and nitrogen atoms provided with the test substrates was incorporated as cellular mass. The lower coefficient observed with asparagine was not due to poor utilization of this substrate, since it readily disappeared from the culture supernatants. However, once taken up, asparagine was not efficiently converted into cellular material.

With respect to the utilization of opines, *Cylindrocarpon* and *Fusarium* species fall into five classes. Strain 4895 grows on octopine only; strains 790 and AR-14 use succinamopine only; strains 88-31 and 86-25 catabolize mannopine and octopine; strains 88-01, 88-02, and 88-03 utilize octopine, octopinic acid, and succinamopine; and strains AR-12, AR-13, and AR-22 grow on mannopine, octopine, and succinamopine. The catabolism of more than one imino diacid opine may reflect a tolerance of opine structural variation by the catabolase system expressed by the opine-utilizing fungi. For *Agrobacterium* spp., mannopine utilization is linked to the ability to degrade agropinic acid and agropine, whereas agropinic acid catabolism is related to the capacity to utilize mannopinic acid (12). In this respect, the catabolism of mannopine by fungi appears similar to that by *Agrobacterium* strains.

Some of the fungi studied showed a combined capacity to catabolize mannopine, octopine, and succinamopine or octopine, octopinic acid, and LL- and DL-succinamopine. If fungi have catabolic enzymes similar to those of bacteria (5, 6), then a particular fungal strain possesses two corresponding classes of degrading enzymes: one mannityl opine and one DL-imino diacid catabolase or one DL-imino diacid and one LL-imino diacid catabolase. Fungi could have evolved by being able to use most of the opine types induced by virulent strains of *Agrobacterium* and consequently to benefit from the ecological niche created by these strains.

Given the smallness of the spore inoculum used, it seems unlikely that complete catabolism of an opine in 8 days or less would result from the selection of spontaneous catabolic mutants. On the contrary, rapid opine catabolism probably results from the activity of the bulk of the spore inoculum and, as such, suggests that this catabolic trait is harbored by natural populations. Another possibility is that the ability to rapidly utilize opines has arisen during isolation or subculturing. This second possibility appears unlikely, because some of the fast opine utilizers have been isolated and maintained on media devoid of opines and nevertheless exhibit growth characteristics similar to those of other strains which have been obtained on an opine-containing selective medium. With some of the strains, a prolonged delay was observed before the complete catabolism of octopine, octopinic acid, or mannopine. This delay could result from poor induction of the catabolic pathway or from the requirement for spontaneous mutation. Such spontaneous mutations to octopine or octopinic acid catabolism have been described for *Pseudomonas* spp. (2, 20).

Cylindrocarpon and *Fusarium* species are well adapted for survival in various environments and are quite versatile nutritionally (3, 4). Opines may represent only a portion of a wide range of compounds used by these fungi as carbon, nitrogen, and energy sources. This situation is similar to that found for opine-utilizing bacteria, such as *Pseudomonas* spp. (20) and coryneform bacteria (23).

Cylindrocarpon and *Fusarium* species are soil-inhabiting fungi and have been reported as saprophytes, weak parasites, and plant pathogens (3, 4). Conceivably, *Agrobacterium* spp. and certain fungi could simultaneously invade plants through wounds. Alternatively, fungal infection could follow *Agrobacterium* infection, and the fungal inhabitants of the crown gall tumor could represent some of the secondary invaders which have been reported to cause gall decay (15). In any case, the significance of the catabolic capacity described here for the process of crown gall tumor colonization by fungi remains to be determined. Similarly, the impact of opines on tumor colonization by the inciting agrobacteria needs to be assessed.

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