APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1978, p. 1150-1154
0099-2240/78/0035-1150$02.00/0
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Vol. 35, No. 6
Printed in U.S.A.

Detection of Mutagens Produced by Fungi with the *Salmonella typhimurium* Assay

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Received for publication 20 December 1977

Forty-one fungal isolates (one isolate per species) representing common plant pathogens and food crop contaminants were grown on sterile, polished rice and assayed for mutagenic activity in the *Salmonella typhimurium*-microsome system. Initially, single doses of aqueous and chloroform extracts of the moldy rice were assayed against the TA100 tester strain by incorporating extracts into the growth medium and by applying small quantities on disks placed on the agar surface. Suspected activity was examined further by analysis of several doses in the plate incorporation assay. Extracts of two aflatoxin-producing isolates (*Aspergillus flavus* and *A. parasiticus*) showed pronounced mutagenic activity, as did extracts of five other isolates (*A. heterothallicus, A. nidulans, A. terricola, Alternaria tenuis,* and *Fusarium moniliforme*) which did not contain detectable aflatoxins. Seven additional isolates (*Botrytis cineria, Ceratocystis fimbriata, Cladosporium herbarum, Fusarium solani* f. sp. *pisi, Penicillium oxalicum, Thermomyces lanuginosus,* and *Verticilium albo-atrum*) revealed activity which was possibly mutagenic; i.e., mutagenic responses were not observed in both the disk and incorporation assays, and clear dose-related activity was not observed in the incorporation assay. Extracts of the remaining fungi were not mutagenic in the bacterial assay.

Acute human poisonings as a result of ingestion of moldy food have been known for centuries (6). Confirmed and suspected acute mycotoxicoses have continued to cause human illness up to the present, even though great improvements have been made in sanitation and storage facilities (2, 10). The possibility that fungi may be involved as etiological agents in chronic diseases, e.g., cancer, was not fully recognized until the early 1960s with the discovery of the aflatoxins. Although the aflatoxins, sterigmatocystin, griseofulvin, luteoskyrin, and cyclochlorotine appear to be the only mycotoxins which have been shown to produce carcinogenic effects upon oral administration of purified substances to animals (8, 12), it is unlikely that these are the only carcinogenic and/or tumorigenic substances produced by fungi.

Relatively little progress has been made in determining the carcinogenic potential of the vast array of fungi with which people may come in contact. In a survey reported by Blank et al. (3), extracts of several fungi pathogenic for humans were shown to produce subcutaneous sarcomas in mice at the sites of injection as well as increased frequency of leukemias and pulmonary tumors. A major obstacle to progress in this area has been the high cost of animal carcinogenesis studies.

Recent refinements of mutagenesis assays and the increasing volume of data which indicate that most chemical carcinogens are also mutagens (1) appear to facilitate and justify a systematic screening of fungi for production of mutagens. Here we report the results of an initial survey of common fungal species, most of which were isolated from food sources, for the production of mutagenic mycotoxins.

The survey was designed to determine: (i) the feasibility of using the bacterial mutagen assay developed by Ames et al. (1) in a large-scale screening of crude fungal extracts, and (ii) the degree to which mutagenic activity occurs in extracts of common fungi.

MATERIALS AND METHODS

Fungi. Fungi were isolated from foods such as walnuts, almonds, pistachio nuts, field corn, cheese, bread, vegetables, and miscellaneous dried fruits. Many isolates were obtained from foods without obvious fungal growth. Nuts, seeds, and small pieces of dried fruit were surface sterilized in 10% NaOCl, rinsed in sterile distilled water for 1 min, and plated on potato dextrose agar (PDA) or rose bengal agar (13). Representative cultures of fungi were transferred to fresh

PDA, purified by single-spore isolation, and tested within 1 month for production of mutagenic compounds. Selected plant-pathogenic fungi from a collection in the Department of Plant Pathology at the University of California, Berkeley, were also tested. Fungi were cultured on PDA slants or plates. Generally, those fungi which sporulated profusely were stored by allowing the slants to dry. Mycelial types or those which did not sporulate readily were transferred monthly. Thermophilic and thermotolerant fungi (*Thermomyces thermophilus, T. lanuginosus, Dactylomyces crustaceus,* and *Mucor pusillus*), generously provided by U. L. Diener of the Auburn University Agricultural Experiment Station, Auburn, Ala., were stored on PDA slants at ambient temperature.

Culture of fungi. For general screening, fungi were grown in cotton-stoppered, 500-ml Erlenmeyer flasks containing 50 g of polished rice which had been moistened with 25 ml of tap water and autoclaved for 15 min at 121°C. (Cracked corn may also be used as substrate.) Flasks were inoculated with aqueous spore or mycelial suspensions from cultures on PDA slants and incubated for 7 to 21 days at 24 to 26°C. Flasks were shaken periodically to ensure homogeneous distribution of inoculum, to minimize clumping of the rice, and to prevent formation of anaerobic areas in the substrate. Thermophilic and thermotolerant species were grown at 50°C in 1-liter flasks on rice medium and on a medium composed of 50 g of shredded wheat and 100 ml of nutrient solution per flask, as described by Davis et al. (4).

Extraction of moldy substrates. Without removal of the cotton plug, chloroform (250 ml) was added to each incubation flask via a long-stemmed funnel. Work area contamination was minimized by careful placement of the funnel stem between the cotton plug and the mouth of the incubation flask. The plug was soaked with an additional portion of chloroform, and the flask was sealed with aluminum foil and allowed to stand at ambient temperature for not less than 12 h. The contents of the flask and an additional 250 ml of water were transferred to a Waring blender jar and blended for 3 min. The mixture was then centrifuged for 15 min at 1,340 × *g* to break up emulsions and was suction filtered. The phases were separated and the aqueous portion was extracted with 100 ml of chloroform. The chloroform was removed from the combined extracts on a rotary evaporator with water aspirator and water bath temperature of 45°C. Aqueous extracts were evaporated to dryness in vacuo with a water bath temperature of 55°C. Extracts were dissolved in 1 ml of dimethyl sulfoxide (Me_2SO) for subsequent mutagen assay. Chloroform extracts were sterile, whereas aqueous extracts often required filter sterilization.

Mutagen assay. The bacterial tester strain, *Salmonella typhimurium* TA100, was obtained from B. N. Ames of the University of California, Berkeley. The bacteria carry the allele *hisG46,* which results in a nutritional requirement for histidine. Mutations which remove the capacity for excision repair and surface lipopolysaccharide synthesis are also present. TA100 also carries the plasmid R factor pKM101. The tester strain was checked, stored, and grown on minimal-glucose agar fortified with traces of histidine and biotin, as outlined by Ames et al. (1). The hepatic S-9 mix was prepared from male Sprague-Dawley rats (250 to 300 g) previously treated with phenobarbital (1). For this general screening, 0.1 ml of S-9 liver preparation was used on appropriate plates. The S-9 liver fraction was included, since some mutagens have been found to be active only after alteration by enzymes present in liver extracts.

Fungal extracts were assayed initially by using the disk method (25 μl of extract applied to 6-mm paper disks and placed on the agar surface) and the plate incorporation method (100 μl of extract per plate). Extracts which produced a ring of revertant colonies around the disk or greater than a 20% increase or decrease in colony count in the plate incorporation method relative to the spontaneous reversion rate of controls were assayed further by testing several doses (10 μl, 50 μl, 0.1 ml, and 0.2 ml) of extract in the incorporation assays. Reversion counts, when less than those of controls, generally indicated toxicity of extracts to bacteria. Growth of the bacterial lawn was invariably reduced relative to controls when a toxic amount of a mutagen was incorporated. A similar series of experiments was carried out for each extract with the inclusion of the S-9 mix. A dose-related response, e.g., increasing numbers of reversions with increasing doses, was assumed to indicate the mutagenicity of the extract.

Each group of assays was accompanied by a series of controls which included individual plates containing TA100 only, S-9 mix only, TA100 plus S-9 mix, TA100 plus Me_2SO (0.1 ml), TA100 plus Me_2SO (0.1 ml) plus S-9 mix, TA100 plus sterigmatocystin (0.1 μg), and TA100 plus sterigmatocystin (0.1 μg) plus S-9 mix. Sterigmatocystin was used as a positive control. Plates were incubated in the dark at 37°C for 2 days, and the numbers of histidine-independent colonies were scored. Representative colonies were occasionally restreaked on minimum agar plus glucose to verify identity. Histidine-independent mutants exhibited good growth and colony characteristics typical of *S. typhimurium.* Typical counts for controls were: 0 (S-9 mix only); 140 to 160 (TA100, TA100 plus S-9 mix, TA100 plus Me_2SO, TA100 plus Me_2SO plus S-9 mix, and TA100 plus sterigmatocystin); and 400 to 500 (TA100 plus sterigmatocystin plus S-9 mix).

RESULTS

The mutagenic activities of extracts of various fungi are summarized in Table 1. Extracts of suspected aflatoxin producers (*Aspergillus flavus* and *A. parasiticus*) showed obvious liver-activated mutagenesis in both the disk and incorporation assays and thus served as positive controls. The presence in these extracts of aflatoxin B_1 was confirmed by the minicolumn–thin-layer chromatography method described by Romer (11). Several other species (*A. heterothallicus, A. nidulans, A. terricola, Alternaria tenuis,* and *Fusarium moniliforme*) also showed definite activity, although neither the aflatoxins

TABLE 1. *Mutagenic activity of fungal extracts*

Species	Source	Mutagenic response with:					
		Disk method (25 µl/disk)		Plate incorporation method			
				100 µl/plate		Multiple doses[a]	
		Water extract	$HCCl_3$ extract	Water extract	$HCCl_3$ extract	Water extract	$HCCl_3$ extract
Alternaria tenuis	Walnuts	−	+	−	+	−	+
Aspergillus awamori	WRRC[b]	±[c]	−	−	−		
A. flavipes	Pistachio nuts	−	−	−	−		
A. flavus[d]	Raisins	−	+	−	+		+
A. heterothallicus	Pistachio nuts	+	−	+	−	±	
A. nidulans[d]	HSU[e]	±	+	+	+	−	+
A. niger	Grapes	−	−	±	−		
A. ochraceous	Pistachio nuts	−	−	−	−		
A. oryzae	Soy sauce	−	−	−	−		
A. parasiticus[d]	USDA[f]	+	+	+	+		+
A. terricola[d]	Pistachio nuts	±	−	+	−	+	−
A. versicolor	Presque Isle[g]	−	−	−	−		
Botrytis cinerea	Grapes	−	±	+	±	±	±
Ceratocystis fimbriata	Sweet potatoes	−	−	−	−		
Cercospora apii	Celery	−	−	−	−		
Cladosporium herbarum	Raisins	±	±	±	±	−	−
Curvularia sp.	Pistachio nuts	−	−	−	−		
Dactylomyces crustaceus	Corn	−	−	−	−		
Fomes annosus	Soil	−	−	−	−		
Fusarium moniliforme[d]	Corn	−	+	−	+	−	+
F. oxysporum f. sp. *cannabis*	*Cannabis sativa*	−	−	−	−		
F. solani f. sp. *pisi*	Pea plants	−	−	−	−		
F. tricinctum	NRRC[h]	−	−	−	−		
Macrophomina sp.	Pistachio nuts	+	−	−	−	−	−
Mucor pusillus	Corn	−	±	−	−		
Penicillium expansum	NRRC[h]	−	−	−	−		
P. oxalicum[d]	Pistachio nuts	−	−	+	+	±	±
P. roquefortii	Cheese	−	−	−	−		
P. urticae	Soil	−	−	−	−		
Penicillium sp.	Walnuts	−	−	−	−		
Phialophora sp.	Pistachio nuts	−	−	+	−		
Pleospora herbarum	Pistachio nuts	−	−	−	−		
Rhizopus stolonifer	Sweet potatoes	−	−	−	−		
Sclerotium rolfsii	Lettuce	−	−	−	−		
Sclerotinia sclerotiorum	Celery	−	−	−	−		
Taphrina deformans	Peach leaves	−	−	−	−		
Thermomyces lanuginosus	Corn	−	−	−	−		
T. thermophilus	Ground feed	−	−	−	−		
Trichoderma viride	Soil	−	−	−	−		
Ulocladium sp.	Pistachio nuts	−	−	+	+	−	−
Verticilium albo-atrum	Cotton plants	−	−	+	+	±	±

[a] Doses of extract used per plate were 10 µl, 50 µl, 0.10 ml, and 0.20 ml.
[b] Western Regional Research Center, Berkeley, Calif.
[c] ±, Results were not clearly positive or negative.
[d] Liver preparation required for activity of extract.
[e] Humboldt State University, Arcata, Calif.
[f] U.S. Department of Agriculture, College Station, Tex.
[g] Presque Isle Cultures, Presque Isle, Pa.
[h] Northern Regional Research Center, Peoria, Ill.

nor sterigmatocystin was detectable in the extracts. A definite dose-response relationship for these extracts confirmed their mutagenic activity. A representative dose-response curve for the chloroform extract of *F. moniliforme* grown on cracked corn is shown in Fig. 1. The decreasing reversion count when doses exceeded 10 µl/plate resulted from the toxicity of the extracts. Possi-

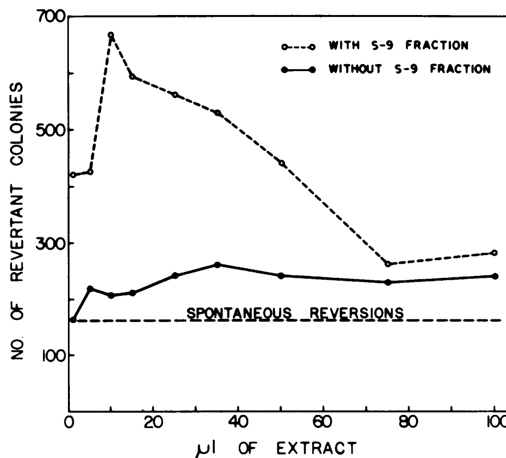

FIG. 1. *Response of S. typhimurium TA100 to various doses of chloroform extracts from F. moniliforme grown on cracked corn.*

ble mutagenic activity was observed in disk or incorporation assays of extracts from seven isolates: *Cladosporium herbarum, F. solani* f. sp. *pisi, Ceratocystis fimbriata, Penicillium oxalicum, Verticilium albo-atrum, T. lanuginosus,* and *Botrytis cineria.* The results are questionable in these cases because positive responses were not observed in both the disk and incorporation assays, nor were clear dose-related responses observed for these extracts. Further testing is required to establish mutagenicity. No mutagenic activity was observed by either method in assays of the remaining fungi.

DISCUSSION

The results of our studies indicate that the Ames *Salmonella*/microsome mutagenicity assay is an efficient and effective means of screening fungi for the production of mutagens. The major time requirement for the total assay is the period necessary for sufficient fungal growth on the selected substrate. Whereas periods of 7 to 21 days were arbitrarily chosen for the present study, other incubation periods may be used for studies of specific phases of the fungal growth cycle. The combination of the disk method with an analysis of a single dose of extract by the incorporation method proved useful in the detection of relatively high mutagenic activity of several fungal extracts. For detection of weakly mutagenic extracts, analysis of several doses by the incorporation method is recommended for all extracts. In all cases, suspected mutagenic activity should be confirmed by establishment of a dose-response relationship for each active extract.

For this initial screening, the tester strain TA100 was used because of its sensitivity to a broad range of mutagens and carcinogens (1). However, many substances are inactive in the TA100 assay and active against other tester strains, e.g., TA98. Thus, the use of strains in addition to TA100 should be considered in more comprehensive screening programs.

The production of mutagens and possibly carcinogens by fungi isolated from common foods is indicative but is not proof of their capacity to produce these metabolites on foods. The monoculture of the fungus on polished rice may be an artificial condition which rarely occurs naturally. Many of the fungi were isolated from foods without obvious fungal growth. Mutagens, therefore, may be present in very small quantities or not at all. These substances are likely to be secondary metabolites which may occur in detectable quantities only after extensive mold growth.

The production of mutagens by an isolate of a given species may indicate that mutagen production is a universal characteristic of all isolates within the species. However, representative isolates would have to be tested to establish this relationship. Conversely, a negative test of only a few isolates does not eliminate the species from consideration as a mutagen producer. This isolate variation in toxin production has been observed, for example, in studies of *Aspergillus* species (9). Thus, to be reasonably certain a given species does not produce mutagens detectable by this assay, many different isolates should be examined.

The conditions for growth of fungi also affect metabolite production. Polished rice was used in this study because it has been shown to be a good substrate for fungal growth and mycotoxin production (9). We also found that extracts of rice did not contain large amounts of interfering substances. Oil obtained from chloroform extractions of corn may interfere with the mutagen assay. However, rice has been reported to be a poor substrate, compared with corn, for production of ochratoxin by *A. ochraceous* (7). Thus, to observe the full potential for toxin production, each isolate should be grown on several substrates. Finally, incubation temperature and humidity are known to affect mycotoxin yields (5). These variables should also be considered when screening fungi for potential mutagens.

For the reasons mentioned above, the screening of fungi for mutagen production is by no means as straightforward as the analysis of purified substances. However, the bacterial mutagen assay developed by Ames et al. (1) is a highly practical method for detecting potentially hazardous fungi. The assay provides an excellent means for determination of which fungi should be subjected to more extensive and costly chemical and biological analyses.

ACKNOWLEDGMENTS

The valuable technical assistance of C.-L. Wu, H. Fukumoto, and M. Morris is gratefully acknowledged.

This work was supported by the California Agricultural Experiment Station and by a Public Health Service Biomedical Sciences support grant administered through the University of California, Berkeley.

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