AN AFLATOXIN STRAIN ATCC-15517

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It is well known that some strains of the genus *Aspergillus* produce aflatoxin and strain ATCC-15517 is one of the first known aflatoxin strains obtained by MATELES (1) from the Tropical Products Institute in London and *A. flavus* LINK ex. FRIES (2) is also the earliest known strain to produce aflatoxin. It seems that the majority of aflatoxin strains, so far as is known, belongs to *A. flavus* and some of them to *A. parasiticus*.

Production of alcoholic beverages and seasonings in Japan has been done mainly by using the fungus *Aspergillus* for over thousand years, and innumerable kinds of strains are known. Since the worldwide occurrence of aflatoxin strains was known, it became one of the most urgent and important problems in Japan where these fungi are being used industrially in this country, although some of strains belonging to *A. flavus* or *A. parasiticus* have rarely been found in some kinds of koji (3).

Taxonomic studies on *Aspergillus* were presented by many workers, such as THOM and CHURCH (4), THOM and RAPER (5), and RAPER and FENNELL (6) on various species of groups, and SAKAGUCHI and YAMADA (7), and OHARA (8) and NEHIRA (9) chiefly on industrial strains in Japan, but no taxonomic studies have hitherto been reported on the aflatoxin strains. Therefore, we examined the strain ATCC-15517 as a representative aflatoxin strain, both from morphological and physiological points, by comparing with about 200 strains of industrial yellow green-spored *Aspergilli*, in which were included mainly *A. oryzae* and its varieties together with some other strains such as 12 strains of *A. flavus*, 24 strains of *A. parasiticus*, and 3 strains of *A. soya*, collected and stocked at the Research Institute of Brewing since 1904, or isolated by the authors from various industrial materials during 1953 to 1962, or given from many factories and laboratories in Japan during 1958 to 1965. The strain ATCC-15517 was received from the National Institute of Health, Tokyo, in 1965.

RESULTS AND DISCUSSION

Taxonomy

Aspergillus parasiticus Speare var. *globosus* Murakami, nov. var.

A specie differt late crescentibus colonies, radiatis, columnaribus capitulis

Fig. 1. Colonies of the strain ATCC-15517 on Czapek solution-agar incubated for 7 days in duplicate at 24° (left) and 34° (right).

conidicis, vesiculosus globosis penitus, 30-40×32-42 μ , et globosis asperulis conidicis, plerumque 4-5 μ in diameter (ATCC-15517, typus).

Aspergillus parasiticus Speare var. *globosus* Murakami, nov. var.

Colonies on Czapek solution-agar incubated for 7 days were velvety, strongly furrowed at both surface and reverse, yellowish yellow green, 40 to 50 mm in diameter at 24° incubation, and dark green with central shades of yellowish brown, 60 to 70 mm in diameter at 34° incubation, as shown in Fig. 1. Such morphological characteristics were not found in any other fungi.

Slant on Czapek solution-agar incubated at 30° began to form conidia in 25 to 30 hr; passing through yellow, 4 to 6 hours after conidia formation, into deep yellow green after 7 days, and keeping very dark greenish gray (MUNSELL notation 5GY-3/2) after over 30 days. Very short stalked, strongly wrinkled transversely, reddish brown pigment forming gradually in agar, sometimes purplish black-colored particles (not sclerotia) scattered at the bottom edge of the tube. Only two strains among the other fungi showed the same color as these in slants, but they were different in other appearances.

Conidiophores 300 to 600 μ long by 6 to 12 μ in diameter with prominently roughened walls. Conidial heads radiate as broom 150 to 200 μ in diameter. Conidiophores broadening upward and acutely enlarging into globose vesicles

Fig. 2. Microstructure of the strain ATCC-15517.

1. Conidial heads
2. Young vesicle
3. Old vesicle
4. Vesicle grown on koji-extract agar

30 to 40 μ by 32 to 42 μ in diameter as shown in Fig. 2. Sterigmata usually in two series, sometimes in one series, tending toward increasing two series by exposure to ultraviolet ray, primary 8 to 10 μ by 4 to 7 μ , secondary 5 to 7 μ by 3 to 4 μ .

Conidia with markedly roughened walls, globose 4 to 5 μ in diameter. Electron micrograms of conidial walls of *A. flavus*, *A. oryzae*, *A. soyae*, *A. parasiticus*, and this fungus are shown in Fig. 3.

The type specimen and living culture derived from the type are deposited in the herbarium of American Type Culture Collection (ATCC-15517; Type).

This variety is very close to *A. parasiticus* but differs from the species in having roughened conidia and entirely globose vesicles. The infraspecific

Fig. 3. Electron micrograms of conidia of various strains.

1. Strain ATCC-15517
2. *A. parasiticus* IAM-2150
3. *A. soyae* IAM-2669
4. *A. flavus* OUT-5004
5. *A. oryzae* OUT-1059

name chosen as descriptive of the vesicular form.

Physiology

Lethal temperature of strains was compared with each other by culturing dry-heat shocked conidia in test tubes, which were obtained from rice-koji extract agar slants incubated at 30° for 7 to 10 days. This strain was weakly resistant to the heating like to many strains belonging to *A. parasiticus* or *A. soyae*, and died out on being heated at 80° for 120 min, at 90° for 30 min, or at 100° for 10 to 20 min. On the contrary, majority of the strains belonging to *A. flavus* and *A. oryzae* were more resistant to the heating and barely died out on being heated at about 100° for 60 to 90 min (see Table 1).

Acid productivities are well known characters of Aspergilli (10). Surface culture of each strain on 50 ml of Ballg. 10 rice koji extract in 100-ml Erlenmyer flask was carried out for 20 days at 30°, and total acid was determined in its filtrates. Acid productivities shown by titers of 0.1 N NaOH per 10 ml of filtrate are given in Table 2. From this point it seems that this fungus is rather akin to *A. parasiticus* than to *A. flavus* and

Table 1. Thermal death time of conidia of yellow green *Aspergilli*.

Strains ^a	Period of heating at 100° (min)	No. of strains killed by heating
<i>A. flavus</i> (12)	60	10
	90	2
<i>A. parasiticus</i> (24)	40<	9
	60	10
	90>	5
<i>A. soyae</i> (3)	40<	3
ATCC-15517 (1)	20	1

^a Numbers in parentheses represent number of strains examined.Table 2. Productivity of acid by yellow green *Aspergilli* cultured on the koji-extract. Acidity is expressed by ml of 0.1 N NaOH/10 ml of filtrate.

<i>A. flavus</i> and <i>A. oryzae</i> group		<i>A. parasiticus</i> and <i>A. soyae</i> group	
Strain No.	Acidity	Strain No.	Acidity
23	0.50	328	1.90
40	2.00	401	1.40
65	1.80	1011	2.70
81	2.00	1023	2.00
128	1.90	1042 ^b	4.50
210	1.50	1044 ^b	1.90
301	1.60	1045 ^b	5.30
513	3.00	1040	2.15
606	0.80	1041	3.60
1020	1.60	1043	1.95
1027	0.80	1046	3.80
1029	1.41	1048	0.50
307 ^a	2.40	1049	5.20
315 ^a	1.20	1050	1.45
331 ^a	1.10	1051	10.70
433 ^a	0.90	1053	1.90
505 ^a	0.60	1054	1.60
609 ^a	1.40	1058	1.20
1028 ^a	0.70	ATCC-15517	10.50
1030 ^a	1.00		

^a *A. flavus* group^b *A. soyae* group

Table 3. Composition of acids produced by yellow green *Aspergilli* shaken cultured in synthetic medium. Amounts of acids produced are expressed by μ moles/liter.

No. of strain	Kojic acid	Lactic acid	Hydroxy-glutaric acid	Succinic acid	Malic acid	Citrmalic acid	Citric acid
69	≡	—	0.06	0.65	0.34	+	0.15
124	—	+	0.01	0.63	0.74	—	0.08
127	—	—	+	0.23	0.63	—	0.14
219	—	0.05	0.05	0.44	0.53	+	0.18
315	++	—	+	0.66	2.06	—	0.13
326	—	—	+	0.11	0.02	—	0.24
330	++	—	0.02	0.53	1.22	—	0.26
411	≡	—	—	0.28	0.28	—	—
433	≡	—	0.01	1.34	2.34	—	0.14
532	≡	—	0.03	1.56	0.19	—	0.09
405 ^a	≡	—	+	0.31	0.03	—	0.06
1011 ^a	±	+	+	1.65	0.29	++	0.05
1042 ^a	≡	+	+	3.56	0.79	—	0.17
1045 ^a	≡	++	0.01	1.01	2.73	+	0.14
1047 ^a	≡	—	—	1.61	0.91	—	0.04
1049 ^a	≡	—	—	1.84	0.62	+	0.03
1050 ^a	≡	+	+	2.87	1.12	—	0.08
1051 ^a	—	+	—	2.69	0.88	—	0.04
ATCC-15517 ^a	≡	—	—	0.12	0.22	+	5.64

^a Strains with remarkably rough-walled conidia.

A. oryzae. By gas chromatographic determination of the medium, main component of acid in this fungus was proved to be citric acid, but succinic acid was found in other fungi, as shown in Table 3.

The predominantly abundant production of blue fluorescence followed by the production of reddish brown pigments is one of the most striking characters of this fungus, but its very weak and sometimes almost lacking amylase activities among many members of yellow green-spored *Aspergilli*, as shown in Table 4, are enough to make this strain an useless fungus in food-making industries. The enzyme activities of fungi in this table were estimated by the two methods reported previously (11). The one is a method using extracts of several small pieces pierced from the center of the Czapek solution-agar plate with a cork borer, in which was cultured a giant colony of the strain by incubation for 5 to 7 days at 34°, and the other is a method using the extract of soy-sauce koji made with a mixture of wheat and soy-

Table 4. Amylolytic and proteolytic activities of yellow green *Aspergilli*.

Strain no. and group	Colonial spot		Soy-sauce koji	
	Amylase	Protease	Amylase	Protease
	D _{30'} ^{40°}	E	D _{30'} ^{40°}	E
67	###	##	302	3.00
81	####	###	684	2.50
83	###	###	499	4.80
127	###	++	509	2.60
216	###	+	415	0.05
333	###	++	434	1.35
609	###	+	571	0.70
328	++	+	32	1.98
401	++	##	32	3.35
405	++	##	52	2.25
410	++	++	23	1.68
1011	++	++	63	1.36
1023	++	##	76	2.90
1024	+	###	13	1.80
1040	++	++	82	1.15
1041	+	—	66	1.35
1043	+	+	61	1.96
1046	++	+	91	3.30
1047	++	##	82	2.20
1048	##	+	221	0.55
1049	+	++	47	1.58
1050	+	—	47	1.08
1051	++	###	85	1.65
1052	+	##	60	1.55
1053	++	++	79	3.30
1054	+	+	58	4.40
1042	++	+	74	3.40
1044	+	++	66	3.90
1045	++	###	71	1.40
ATCC-15517	+	##	50	2.75

beans. The amylolytic and proteolytic activities measured by these methods were shown respectively by D_{30'}^{40°} (Wohlgemuth value) and by E (extinction at 660 mμ in solution in which the enzyme was acted on casein).

It is very interesting that the *A. soyae* strains, which have been used

for soy-sauce making in Japan, have smooth conidiophores but prominently roughened conidia and very strong protease activities, while *A. parasiticus* strains, which have rarely been used for that, have both prominently roughened conidiophores and conidia, and their protease activities are not stronger than those of *A. soyae* or *A. oryzae* and *A. flavus*.

Several physiological characters of this fungus were compared with those of other fungi by the previously reported method (12-14), in which strain ATCC-15517 was denoted as no. 1055. The activity of this fungus was positive in reducing Methylen Blue or Triphenyltetrazolium chloride but almost negative in coloring phenols such as hydroquinone or pyrogallol, and browning of rice-koji made by this fungus by air scarcely occurred. However, these properties cannot be taken as specific to this fungus, because these characters vary from strain to strain, and sometimes with conditions of experiments.

Mutation

Mycological relations between this fungus and other fungi were considered by comparing the mutants derived from this fungus with other fungi, and by testing variation in the aflatoxin-producing ability among the mutants. A certain number of conidia of this strain was rubbed and scattered on the surface of Czapek solution-agar plate, then exposed to ultraviolet rays of 2,537 Å emitted from 15-w UV-lamp, keeping various direct distances from the medium and various hours of exposure, cultured for several days, and 5 kinds of mutants, named B, D, E, G, and I, were obtained. Microstructure and its explanation in addition to exposure condition are shown in Fig. 4 and Table 5, respectively. Strain ATCC-15517, namely, the parent strain, resisted ultraviolet rays more strongly than other fungi. For example, the former survived even after 8 to 10 min exposure without any variation, but the latter died out or gave rise to a mutant after 4 to

Table 5. Conditions for exposure to ultraviolet irradiation and characteristics of mutants derived from *A. parasiticus* ATCC-15517.

Mutant strain	Condition of UV irradiation		Type of mutant	Arrangement of sterigmata
	distance (cm)	time (min)		
B	30	8	albino	80% in one series, 20% in two series
D	30	15	yellow green	80% to 100% in two series
E	30	15	pigment less	90% in elongation, 10% in one series
G	30	15	yellow brown	all elongated and enlarged
I	30	13	restricted	80 to 90% in two series

Fig. 4. Microstructure of the mutants B, D, E, G and I, all derived from the strain ATCC-15517.

1. Mutant B, conidial heads are very small.
2. Mutant B, vesicles are small but globose form similar to those of the parent.
3. Mutant D, conidial heads are the same as those of the parent.
4. Mutant D, vesicles are the same as those of the parent.
5. Mutant E, conidial heads are small and their forms are different from the parent.
6. Mutant E, vesicle with elongated sterigmata in two series is seen.
7. Mutant G, vesicles with elongated and enlarged sterigmata in two series are seen.
8. Mutant I, conidial heads are the same as those of the parent.
9. Mutant I, vesicle is the same as that of the parent.

Table 6. Detection and determination by thin-layer chromatography and fluorophotometry of aflatoxin produced by the strain ATCC-15517 and its mutants.

Mutant and parent strain	Dry weight of fungus (g)	Chromatogram spot						Relative value of fluorescence energy corresponding to various wave lengths ($m\mu$)					
		B ₁	G ₁	X	B ₂	G ₂		E ₁ ^a	A ₁ ^b	RE ₁ ^c	E ₂ ^a	A ₂ ^b	RE ₂ ^c
B	0.285	++	++	+	##	+		372	426	1462	470	526	102
D	0.547	####	####	##	##	##		386	425	21060	469	524	965
E	0.336	##	##	##	##	##		376	424	10223	470	526	405
G	0.387	##	##	##	##	##		374	424	3228	470	526	238
I	0.902	##	##	##	##	##		385	426	5963	470	526	781
ATCC-15517	0.982	##	##	##	##	##		382	424	10180	470	524	102

^a Excitation wave length.

^b Emission wave length.

^c RE denotes relative value of fluorescence energy. For value (arbitrary unit) see text.

6 min exposure, both at an equal distance of 30 cm from the medium.

It was observed that mutants varied especially in the arrangement followed by deformation of sterigmata, and sometimes conidial heads were dwarfed, but no variation occurred in wrinkles at colonial reverse, globose shape of vesicles, and roughness of conidiophores and conidia. Therefore, no continuous relations were found among strain ATCC-15517 and other various kinds of strains, at least morphologically, and an independent position of this strain should be supported.

Aflatoxin-producing abilities were compared among these mutants and their parent strain. Strains were cultured in 80 ml of medium (1) by shaking at 30° for 76 hr, and aflatoxin mixture was obtained from the culture filtrate by repeated treatment with chloroform and petroleum ether followed by dissolution in chloroform. Aflatoxin was detected by thin-layer chromatography and determined by spectrofluorophotometer. Total mass of fungi was weighed as dry matter. Results are shown in Table 6, in which signs from + to #### show the fluorescence intensities of the spots corresponding to their concentrations from weak to strong in that order. Normal components of aflatoxin B₁, G₁, B₂, and G₂ are listed, but X is an unknown, blue fluorescent spot found additionally, in which mutants D, E, and G show some brown shaded fluorescence. E and A in the table respectively indicate the excitation wave-length and emission wave-length. The relative value of fluorescence energy indicated by RE₁ and RE₂ were comparatively determined from the value of 2,000 shown by 1 ppm of quinine sulfate solution and that of 1,500 shown by 1 ppm of riboflavin solution, respectively, and both were

calculated per one gram of dry matter of fungus. Usually, RE₁ and RE₂ respectively show the concentration of blue and green fluorescent substances contained.

The blue fluorescence decreased except for mutant D, in which it increased about two-fold, and the green fluorescence increased in every mutant. From this point of view, strain ATCC-15517 is one of the ordinary yellow green-spored *Aspergilli* whose blue fluorescence productivity is specially enhanced.

SUMMARY

In an attempt to clarify the taxonomic situation of the strain ATCC-15517, which is a well-known aflatoxin producing fungus, its general morphological and physiological characters, in addition to mutation by ultraviolet rays, were examined by comparing with many kinds of industrial strains used in Japan.

From the results obtained, it seems that this fungus belongs to *A. parasiticus* rather than to *A. flavus* because of its remarkably rough walled conidia and conidiophores, sensitivity to heating, high productivity of acid, strong resistance to ultraviolet light, and other physiological observations, excluding its unstable arrangement of sterigmata.

Thus, it should be placed in a new strain of *A. parasiticus* series by its globose-shaped vesicles: *A. parasiticus* Speare var. *globosus* MURAKAMI nov. var.

Finally, it was confirmed that neither this fungus nor its allied strain was found at all in Japanese industrial strains, from the point of morphology.

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