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Incidence of *Alternaria* Species in Red Pepper and Sesame from Korea and Their Ability to Produce Mycotoxins

Hyang Burm Lee and Seung Hun Yu*
Department of Agricultural Biology, College of Agriculture, Chungnam National University, Taejon 305-764, Korea

한국산 고추와 참깨에 발생하는 *Alternaria*의 종류와 이들의 진균독소 생성능력

이향범 · 유승헌*
충남대학교 농생물학과

ABSTRACT : ***Alternaria alternata*** **and** ***A. solani*** **were identified from 130** ***Alternaria*** **isolates obtained from red pepper fruits, and three species including** ***A. alternata*****,** ***A. sesami*** **and** ***A. sesamicola*** **were detected from 150 isolates from sesame seeds. Among the 4** ***Alternaria*** **species,** ***A. alternata*** **was the predominant fungus from both plants, having incidence of 95.4% in red pepper and 56.0% in sesame. Of the total 280 isolates, cultures on autoclaved rice of 75 isolates were tested for toxicity to 21-day-old virgin female rats. Out of 50 isolates of** ***A. alternata*****, 17 were lethal to rats, inducing congestion and hemorrhage of stomach and intestine and kidney enlargement, and 8 caused lack of weight gain or weight loss. The other 25 isolates of** ***A. alternata*** **and all the isolates of** ***A. sesami*****,** ***A. sesamicola*** **and** ***A. solani*****, showed no significant indication of toxicity. Production of mycotoxins in the rice cultures of the above 75 isolates belonging to 4 species was analyzed.** ***Alternaria*** **cultures were extracted with methanol and purified by using solvent partition, thin-layer chromatography, and high performance liquid chromatography. Of the four species tested, all produced alternariol (AOH) and alternariol monomethyl ether (AME), three (*****A. alternata*****,** ***A. sesami*** **and** ***A. sesamicola*****) produced altenuene (ALT) and altertoxin-I (ATX-I), and only** ***A. alternata*** **produced tenuazonic acid (TA). TA was produced by all of the highly toxic (lethal to rats) isolates of** ***A. alternata*****, but not by any nontoxic isolates.**

Key words **:** ***Alternaria*** **mycotoxins,** ***Alternaria alternata*****,** ***A. sesami*****,** ***A. sesamicola*****,** ***A. solani*****.**

Alternaria molds are one of the most common microorganisms found in the agricultural ecosystem. The genus includes both plant pathogenic and saprophytic species that may damage crops in the field or cause postharvest decay of fruits and vegetables. Because the molds can grow at low temperatures, they are often involved in the spoilage of refrigerated products (10, 24). Some are infesting and often parasitizing seeds of a wide variety of crops.

Certain *Alternaria* species are also capable of producing mycotoxins (6, 17) which can contaminate agricultural products (14, 20, 24). Five major *Alternaria* toxins belong to three different structural classes: alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT) which are benzopyrone derivatives; altertoxin-I (ATX-I) which is a perylene derivative; and tenuazonic acid (TA), a tetramic acid derivative. A number of reviews on *Alternaria* toxins dealing with different aspects such as biosynthesis and bioregulation (23), chemical properties and analytical methods (19, 21), toxicity and assessment of plant product contamination (12,26) have been published.

Red pepper fruits with *Alternaria* black rot are frequently observed in several localities in Korea at harvest time (11). The *Alternaria* black rot may

*Corresponding author.

also appear during storage of the red pepper fruits. Sesame seeds are reported to be infested by several fungal genera including *Alternaria*, *Corynespora* and *Fusarium*, but the major mold contaminants of the seeds are *Alternaria* species (27).

The purpose of this study is to determine the predominant *Alternaria* species that occur in red pepper fruits and sesame seeds, to evaluate their toxicity to rats and their ability to produce *Alternaria* mycotoxins under laboratory conditions.

MATERIALS AND METHODS

Red pepper and sesame. A total of 150 fruits of red pepper with black mold were collected from different farmers' fields in the Chungnam and Chungbuk Provinces during August 1992 and 1993. Twenty five samples of sesame seeds were collected from different farmers' stock in the Chungnam and Kyungbuk Provinces during September 1992 and 1993. All samples were stored at 5℃ until use.

Incidence and isolation of *Alternaria* species. Tissue pieces were removed from the decayed parts of red pepper fruits with a razor blade, soaked in 2% NaOCl solution for 1 min, rinsed in sterile distilled water, transferred to water agar medium supplemented with 10 mg of streptomycin sulfate per liter, and incubated at 25℃ for 5~7 days under ultraviolet lamps (12 h photoperiod). For the isolation from sesame seeds, two-hundred seeds per sample were plated on water agar and incubated under the same conditions described above. The plates were observed under a stereomicroscope, and three single-conidial cultures, representing each suspected *Alternaria* colony, were transferred to potato dextrose agar (PDA). Colonies were incubated at 25℃ for 7~10 days, and then identified using the classification systems of Ellis (4) and Yu (28). In particular, growth and color of the colonies were observed on PDA, and morphological characteristics of conidia and conidiophores were observed on PDA or sometimes on tissue pieces of red pepper fruits or sesame seeds.

Preparation of cultures. *Alternaria* isolates were cultured on rice in order to evaluate their toxigenic potential. Rice kernels (200 g) were adjusted to about 45~50% moisture content in 500 ml Erlenmeyer flasks, and autoclaved at 120℃ for 20 min. Each *Alternaria* isolate was inoculated on the rice medium, incubated at 25℃ for 3 weeks in the dark, and then dried at 60℃ for 48 h.

Rat feeding test. The moldy rice was ground to powder in a Waring blender. Female Sprague-Pawley rats, 21-day-old and weighing approximately 50 g each were obtained from the Experimental Animal Farm, Seoul National University. They were housed in individual cages and fed a 1 : 1 mixture of ground moldy rice and a basal diet in powder form. The animals were weighed at the beginning and at the end of the experiment. Three rats were used for each fungal isolate and three control rats that received control diet only were also used. They were observed frequently for 10 days, and major symptoms and death were recorded. Surviving rats were sacrificed by diethyl ether inhalation and examined for pathological changes of internal organs.

Extraction of *Alternaria* cultures. Each ground rice culture (40 g) was extracted in a blender with 80 ml of methanol, and the extract was filtered through Whatman no. 2 filter paper. Forty ml filtered solution was stirred with 80 ml of 20% aqueous ammonium sulfate, and filtered. One hundred ml of the filtrate were extracted twice with 5 ml of methylene chloride, and the combined extracts were evaporated to dryness. The residue was dissolved in 1 ml of methanol and analyzed for AOH, AME, ALT and ATX-I by TLC and HPLC.

For the analysis of TA, the aqueous solution left after methylene chloride extraction was acidified (pH 2) with concentrated hydrochloric acid and extracted twice with 50 ml methylene chloride. TA was extracted from the combined organic phases with 30 ml of 5% aqueous sodium bicarbonate, then reconverted to its acidic form by adjusting to pH 2 with 1 N hydrochloric acid, and extracted twice with 30 ml of methylene chloride. The methylene chloride extracts were combined, washed with 25 ml water, and evaporated to dryness. The residue was made up to 2 ml with methanol and analyzed by TLC and HPLC according to procedures described by Visconti *et al.* (25).

TLC and HPLC. Two dimensional TLC analysis was performed on silica gel pre-coated plates with a fluorescence indicator by using toluene-ethyl acetate-90% formic acid (60 : 30 : 10, v/v/v) and chloroform-acetone (88 : 12, v/v) as solvent systems. Resolution (R_f values) of the different toxins is reported in Table 1. AOH and AME were visualized under long wavelength of UV light by their blue fluorescence. Fluorescence quenching spots were observed for AOH, AME, ALT, ATX-I and TA on plates irradiated with short wavelength of UV light.

Isolation of the mycotoxins for the subsequent HPLC analysis was performed by TLC chromatography of the extracts with the toluene-ethyl acetate-90% formic acid solvent system as above and elution of the separated layers with methanol.

Standards of *Alternaria* mycotoxins were kindly supplied by Dr. Solfrizzo, M., Instituto Tossine e Micotossine da Parassiti Vegetali, Consiglio Nazionale delle Ricerche, 70126 Bari, Italy.

HPLC analysis was used both for conformation of TLC results and for quantitation of AOH, AME, ALT, ATX-I and TA. The apparatus was a Waters Model 452 equipped with a variable wavelength spectrophotometric detector. The chromatographic separation was performed on a reverse phase column (Bondapak C_{18}). Methanol-water (60 : 40, v/v) was used as mobile phase for the analysis of ALT; methanol-water (80 : 20, v/v) containing 300 mg $ZnSO_4 \cdot 7H_2O$/l for AOH, AME, and TA; methanol-water (60 : 40, v/v) containing 0.1 M $NaNO_3$, and 0.001 M $HN0_3$ for ATX-I. The wavelength of the UV detector was set at 280 nm for detection of ALT and TA, and at 257 nm for AOH, AME and ATX-I. Retention times are reported in Table 1.

Table 1. TLC R_f values and HPLC retention times of *Alternaria* mycotoxins

Toxin[a]	TLC[b] R_f		HPLC[c] retention time (min.)
	A	B	
AOH	0.34	0.45	4.80
AME	0.62	0.64	6.19
ALT	0.08	0.17	6.26
ATX-I	0.17	0.36	7.02
TA	0.04	0.39	5.95

[a] AOH = alternariol; AME = alternariol monomethyl ether; ALT = altenuene; ATX-I = altertoxin-I; TA = tenuazonic acid.

[b] TLC was performed on silica gel pre-coated plates with a fluorescence indicator by using chloroform-acetone (88 : 12, v/v) (A) and toluene-ethyl acetate-90% formic acid (60 : 30 : 10, v/v) (B) as solvent systems.

[c] HPLC was performed on a reverse phase column (Bondapak C_{18}). Methanol-water (60 : 40, v/v) was used as mobile phase for the analysis of ALT; methanol-water (80 : 20, v/v) containing 300 mg $ZnSO_4 \cdot 7H_2O$/l for AOH, AME, and TA; methanol-water (60 : 40, v/v) containing 0.1 M $NaNO_3$, and 0.001 M HNO_3 for ATX-I. The wavelength of the UV detector was set at 280 nm for detection of ALT and TA, and at 257 nm for AOH, AME and ATX-I.

RESULTS

Incidence of *Alternaria* species. Two *Alternaria* species, *A. alternata* and *A. solani* were identified from 130 isolates obtained from 150 red pepper fruits, having incidence of 95,4%, and 4.6%, respectively (Table 2). In sesame seed samples, 3 species were detected from 150 isolates: *A. alternata* (56.0%); *A. sesami* (12.0%); and *A. sesamicola* (32.0%). The predominant *Alternaria* species isolated from both red pepper fruits and sesame seeds was *A. alternata.*

Toxicity of *Alternaria* isolates. Of the 280 *Alternaria* isolates identified, 75 isolates were prepared and examined for toxicity by feeding *Alternaria*-infested rice to rats.

Seventeen (12 from red pepper and 5 from sesame) of the 50 isolates of *A. alternata* resulted in death of the rats that consumed the fungus-infested feeds within 3~9 days (Table 3). All of the rats that succumbed to the *Alternaria*-infested rations lost weight. The major clinical signs of the dead rats were stomach congestion and hemorrhage, intestine congestion and hemorrhage, and kidney enlargement. Eight isolates of the *A. alternata* that were not lethal to rats in 10 days, caused lack of weight gain or weight loss during the test period. All of the isolates of *A. sesami*, *A. sesamicola* and *A. solani* were not lethal to rats or showed no toxigenic indications to the rats in the feeding test. Most of the

Table 2. Incidence of *Alternaria* species in red pepper fruits and sesame seeds

Alternaria species	Incidence (%)[a]	
	Red pepper (130)[b]	Sesame (150)[b]
A. alternata	95.4	56.0
A. sesami	0.0	12.0
A. sesamicola	0.0	32.0
A. solani	4.6	0.0

[a] Red pepper fruits with *Alternaria* black mold and sesame seeds were investigated.

[b] The total number of *Alternaria* isolates specified.

rats that fed ground cultures of *A. sesami*, *A. sesamicola* and *A. solani* gained normal weight and consumed approximately as much feed as the controls.

Production of mycotoxins. Production of mycotoxins in the rice cultures of the 75 *Alternaria* isolates belonging to the 4 species detected in this study is shown in Table 4.

Toxin production was different among isolates in a same species and among *Alternaria* species identified. All of the *Alternaria* isolates except one of *A. sesami* produced at least one toxin. Eighteen isolates (13 from red pepper and 5 from sesame) included in *A. alternata* produced all 5 toxins, of which TA was produced mainly by these isolates. TA was not produced by other *Alternaria* species than *A. alternata*. Nearly all isolates of *A. alternata* produced AOH and AME; whereas 46 and 47 out of 50 isolates produced ALT and ATX-I, respectively. *A. sesami* and *A. sesamicola* produced the 4 toxins: AOH, AME, ALT and ATX-I. Nearly all isolates of the two species also synthesized both AOH and AME; whereas only 2 out of 10 *A. sesami* isolates produced ALT and ATX-I, which were produced by 8 out of 10 *A. sesamicola* isolates. In case of *A. solani*, 4 and 3 out of 5 isolates produced AOH and AME,

Table 3. Toxicity to rats of *Alternaria* species isolated from red pepper and sesame[a]

Alternaria species	Source	No. of isolates		Days to death
		Tested	Lethal	
A. alternata	Red pepper	30	12	3~9
	Sesame	20	5	4~9
A. sesami	Sesame	10	0	
A. sesamicola	Sesame	10	0	
A. solani	Red pepper	5	0	
Total		75	17	

[a] Isolates were grown on autoclaved rice kernels at 25℃ for 3~4 weeks, and the fungus-infested grain was dried, ground and fed a 1 : 1 mixture of ground moldy rice and a basal diet in powder form to 21-day-old female white rats for 10 days.

Table 4. Production of mycotoxins by four *Alternaria* species isolated from red pepper and sesame under laboratory conditions[a]

Alternaria species	Source	No. of isolates tested	No. of positive isolates	Toxin production[b]				
				AOH	AME	ALT	ATX-I	TA
A. alternata	Red pepper	30	13	+	+	+	+	+
			15	+	+	+	+	−
			1	−	+	+	+	+
			1	+	+	−	−	−
	Sesame	20	5	+	+	+	+	+
			11	+	+	+	+	−
			1	+	+	+	−	−
			1	+	+	−	−	+
			1	+	+	−	+	−
			1	+	−	−	+	−
A. sesami	Sesame	10	2	+	+	+	+	−
			5	+	+	−	−	−
			1	+	−	−	−	−
			1	−	+	−	−	−
A. sesamicola	Sesame	10	7	+	+	+	+	−
			1	+	+	+	−	−
			1	+	+	−	+	−
			1	+	+	−	−	−
A. solani	Red pepper	5	2	+	+	−	−	−
			1	−	+	−	−	−
			2	+	−	−	−	−

[a] Each isolate was grown on autoclaved rice at 25℃ for 3 weeks.
[b] Determined by TLC. AOH=alternariol; AME=alternariol monomethyl ether; ALT; altenuene; ATX-I=altertoxin-I; TA=tenuazonic acid; +=mycotoxin was detected; −=mycotoxin was not detected.

Table 5. Profile of mycotoxins produced by 20 isolates of *Alternaria alternata* and their toxicity to rats

Isolate number	Source	Mycotoxin production (mg/kg)[a]					Toxicity to rats[b]
		AOH	AME	ALT	ATX-I	TA	
R-1	Red pepper	23	30	7	20	1926	L[c]
R-2		1	42	4	1	ND	NS
R-3		ND	5	4	2	1733	L
R-4		108	65	2	1	3420	L
R-5		5	8	ND	ND	ND	NS
R-6		70	512	46	11	1858	L
R-7		65	17	17	25	ND	BWL
R-8		38	12	7	4	ND	NS
R-9		97	54	3	17	2600	L
R-10		20	5	4	2	ND	NS
S-1	Sesame	30	65	35	4	ND	NS
S-2		55	27	ND	ND	550	BWL
S-3		28	76	3	5	ND	NS
S-4		20	1	10	8	950	L
S-5		35	22	14	ND	ND	NS
S-6		59	17	7	5	ND	BWL
S-7		65	30	ND	10	ND	NS
S-8		20	42	4	2	1870	L
S-9		60	78	17	1	ND	BWL
S-10		5	3	2	4	ND	NS

[a] Determined by HPLC. AOH=alternariol; AME=alternariol monomethyl ether; ALT=altenuene; ATX-I=altertoxin-I; TA=tenuazonic acid; ND=not detected.
[b] Each isolate was grown on autoclaved rice at 25℃ for 3~4 weeks, and the fungus-infested grain was dried, ground and fed a 1 : 1 mixture of ground moldy rice and a basal diet in powder form to 21-day-old female white rats for 10 days.
[c] L=lethal; NS=no symptom; BWL=body weight loss.

respectively, and none of the isolates produced TA, ALT and ATX-I.

The profile of mycotoxin production in the rice cultures of twenty isolates of *A. alternata* isolated from red pepper and sesame is reported in Table 5. The benzopyrone (AOH, AME, and ALT) and perylene (ATX-I) compounds were synthesized by most of the isolates. The highest amounts of benzopyrone derivatives produced were 108, 512, and 46 mg/kg of AOH, AME and ALT, respectively. The highest amount of ATX-I produced was 25 mg/kg. TA was produced only by 8 isolates of the *Alternaria* and the amount of TA ranged between 550 and 3420 mg/kg. All of the 7 highly toxic (lethal to rats) isolates produced TA, and 9 nontoxic isolates produced only benzopyrone and perylene derivatives.

One isolate producing the lowest amount of TA among the 8 TA-producing isolates showed no lethal effect but caused weight loss.

DISCUSSION

Our findings indicate that the major *Alternaria* species isolated from red pepper fruits with black mold and sesame seeds from Korea is *A. alternata* but that other *Alternaria* species are present at lower levels in and on these agricultural products. *A. alternata*, the most common species, is widely distributed in plants and geographic regions (5). This species is known to be commonly associated with *Alternaria* rot of apples (24), tomatoes (24), mandarins (15) and red peppers (12). A survey of small grains conducted in the United States showed that *A. alternata* was the major species present, along with other less common species (1). Our results also support the cosmopolitan distribution of *A. alternata*.

Many members of *Alternaria* can produce toxic metabolites in feed and food stuffs they infest. Forgacs *et al.* (5) found that grain molded by an *Alter-*

naria isolate caused death with hemorrhagic syndrome in 19 of 20 chicks by the 3rd and 4th day. Christensen *et al.* (2) using fungi isolated from foods and feeds, many from feeds suspected to be toxic, showed that 78% of the *Alternaria* isolates (65 of 83) grown on a grain medium were lethal to rats fed the infected grain. In another study with fungi isolated from noncured tobacco, about the same proportion of isolates of *Alternaria* (30 of 38) and *Penicillium* (29 of 37) were toxic when the homogenized cultures were injected into mice (8). An isolate of *A. alternata* was reported to be as toxic as *Penicillium steckii*, and other *Alternaria* isolates were as toxic or more toxic than extracts of another 13 genera of molds when tested against brine shrimps, chick embryos and rats (3). In contrast, Gupta *et al.* (7) reported that *Alternaria* culture extracts were less toxic in mice by injection than those of *Aspergillus*, *Chaetomium*, *Fusarium* or *Penicillium* spp. However, their pretreatment of the extract may have destroyed some of the toxins that are heat sensitive. Hence, it seems that *Alternaria* must also be considered an important genus of toxic molds. The results of our study also indicate that highly toxic isolates are distributed in the isolates of *Alternaria*. But the toxic isolates were found only in the species of *A. alternata*, not in the isolates of *A. sesami*, *A. sesamicola* and *A. solani*.

The results of our study proved that there are qualitative as well as quantitative differences in toxin production among *A. alternata* isolates. Also, there were differences in the toxicity of *A. alternata* cultures, suggesting that the toxicity is related to toxin production. In the study of Sauer *et al.* (18), grains molded by a number of different *Alternaria* isolates were analyzed for toxin content. Although several toxins were found in the molded grains, only the diets containing TA showed toxicity.

Pero *et al.* (17) concluded that all three classes of *Alternaria* toxins they evaluated (the dibenzopyrones, the tetramic acid and the altertoxins) have some degree of toxicity to mammalian and bacterial cells as well as to mice. In their study, AOH and AME appear to be much less toxic to mice than TA and ATX, although a combination of AOH and AME are synergistically toxic to *Bacillus* sp. Meronuck *et al.* (16) suggested that mammalian toxicity of *A. alternata* was due primarily to TA, because they found that 57 of 85 (67%) isolates of *A. alternata* from plant materials were lethal to rats fed a corn-rice substrate on which these isolates were grown. Of 23 toxic isolates analyzed for TA and AME, 20 produced TA, whereas 14 produced AME. By comparison, none of the 11 nontoxic isolates examined produced TA, and two produced AME. Harvan and Pero (9) in their review on the *Alternaria* metabolites reiterated the conclusion of Meronuck *et al.* (16) that TA is the major mammalian toxin produced by *Alternaria* spp. Smith *et al.* (22) described well the toxic effect of single and repeated doses of TA on mice, rats, dogs, and monkeys. The single oral dose LD_{50} of the sodium salt of TA in rats was 174 mg/kg, whereas in dogs repeated daily oral doses of 10 mg/kg (4 doses of 2.5 mg/kg) caused death in 8 or 9 days. TA salt was much better tolerated by monkeys than by dogs; monkeys tolerated for 30-day doses, two to four times the level of TA salt that killed 1 of 2 dogs in 18 days. Our results also suggest that lethal toxicity of *A. alternata* is due primarily to TA, because all the 7 highly toxic isolates produced TA and none of the 9 nontoxic isolates examined produced TA.

However, Meronuck *et al.* (16) concluded it is unlikely that there is a serious hazard of acute toxicity to humans from TA in foods because relatively large doses of TA are required to produce acute toxic effect (100 to 200 mg/kg to be lethal to rats) and especially there is little evidence for its natural occurrence in foods. Harvan and Pero (9) also suggested that only in underdeveloped nations would there be much possibility of human exposure to acutely toxic doses but that there is a substantial possibility of exposure to low levels causing chronic toxicity. Hence, it seems very likely that chronic toxicity should be the main concern for *Alternaria* toxins rather than acute toxicity in much the same way that carcinogenicity and chronic toxicity are the primary concern for the aflatoxins and most of other mycotoxins. A recent work (13) showed that AOH and AME produced by *A. alternata* play an important role in the etiology of human esophageal cancer.

Our findings in this study indicate that a potential mycotoxin problem may exist in some agricultural products due to the presence of *Alternaria* species. This is the first report of the production of AOH, AME, ALT, and ATX-I by the species of *A. sesami* and *A. sesamicola*, and of AME by *A. so-*

lani. Further research on the natural occurrence of *Alternaria* mycotoxins in some agricultural products is under way.

요 약

고추와 참깨에서 총 280개의 *Alternaria* 균주들을 분리하였다. 고추에서 2종의 *Alternaria*(*A. alternata*와 *A. solani*)가 동정되었고 참깨에서는 3종의 *Alternaria* (*A. alternata*, *A. sesami*, *A. sesamicola*)가 검출되었으며 *A. alternata*가 고추와 참깨 모두에서 우점종이었다. 독성실험을 위하여 75개 *Alternaria* 균주들의 쌀배양체를 준비하여 생후 21일된 흰쥐 암컷에 사료와 섞어 먹인 후 그 증상들을 관찰하였다. *A. alternata* 50개 균주 중 17개 균주가 쥐를 치사시켰으며 위와 장의 출혈과 신장 비대현상을 일으켰다. 또한 8개 균주는 쥐를 치사시키지는 않았으나 체중을 증가시키지 않거나 감소시켰다. 그리고 *A. sesami*, *A. sesamicola*, *A. solani* 균주들 중에는 쥐를 치사시키는 균주가 없었다. 검출된 4종의 *Alternaria* 75개 균주를 사용하여 쌀배양체내에서의 진균독소 생성능을 조사하였다. *Alternaria* 배양체를 메탄올로 추출하고 용매분획과 TLC 및 HPLC 분석을 이용하여 순화하였다. 이 연구에 사용된 4종의 *Alternaria* 중에서 모든 종들이 alternariol(AOH)과 alternariol monomethyl ether (AME)를 생성하였으며 3종(*A. alternata*, *A. sesami*, *A. sesamicola*)이 altenuene(ALT)과 altertoxin-I(ATX-I)을 생성하였고, 단지 *A. alternata*만이 tenuazonic acid(TA)를 생성하였다. 쥐를 치사시킨 모든 *A. alternata* 독성균주들은 TA를 생성하였으나 비독성균주들은 모두 TA를 생성하지 않았다.

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