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Production of Secondary Metabolites by Some Terverticillate Penicillia on Carbohydrate-Rich and Meat Substrates

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ABSTRACT

Most terverticillate penicillia isolated from dry-cured meat products are toxigenic, but their ability to produce hazardous metabolites on meat-based substrates is not well known. The production of extrolites by selected terverticillate penicillia isolated from dry-cured ham has been studied on carbohydrate-rich media (malt extract agar, Czapek yeast autolysate agar, rice extract agar, and rice), meat extract triolein salt agar, and ham slices. Chloroform extracts from the selected strains grown on malt extract agar were toxic for the brine shrimp (*Artemia salina*) larvae and VERO cells at a concentration of 2 mg/ml, but 0.02 mg/ml produced no toxic effect. Analysis by high-pressure liquid chromatography (HPLC) coupled with photodiode array detection (DAD) or with mass spectrometry (MS) and an atmospheric pressure chemical ionization (APCI) source revealed different biologically active metabolites: cyclopiazonic acid and rugulovasine A from *Penicillium commune*; verrucosidin, anacine, puberuline, verrucofortine, and viridicatols from *Penicillium polonicum*; arisugacin and viridicatols from *Penicillium echinulatum*; and compactin and viridicatols from *Penicillium solitum.* Most of these metabolites, including the amino acid–derived compounds, were produced in the media containing high levels of carbohydrates. High concentrations of nitrogen compounds in the medium does not imply a greater production of the metabolites studied, not even those derived from the amino acids. However, molds growing on dry-cured ham are able to synthesize limited amounts of some secondary metabolites, a fact not previously reported. The combination of HPLC coupled with DAD and MS-APCI was useful for identification of closely related terverticillate *Penicillium* species from dry-cured ham. These techniques could be used to characterize the risk associated with the potential production of secondary metabolites in cured meats.

Ripening and storage conditions of most dry-cured meat products favor the development of an uncontrolled superficial fungal population. Among the molds, terverticillate penicillia (subgenus *Penicillium*) dominate during most of the ripening process *(1, 25, 35, 44).* Different species of *Penicillium* contribute to texture and flavor development of dry-cured meat products by lowering levels of lipid oxidation products, increasing proteolysis, and producing specific volatile compounds *(6, 29, 30).* For these reasons, molds are considered a desirable component of the wild microbial population on these products.

However, several species of *Penicillium* isolated from meat products produce mycotoxins *(1, 11, 14, 44).* Chloroform extracts from terverticillate penicillia isolated from dry-cured ham and grown on malt extract agar and Czapek yeast autolysate agar were toxic to brine shrimp (*Artemia salina*) larvae and VERO cells *(35).* Some of these molds also synthesize various mycotoxins and other potentially toxic secondary metabolites, such as viridicatol-related alkaloids *(1, 17, 25, 44).* Nonetheless, the production of mycotoxins and other fungal metabolites in foods depends on the characteristics of the substrate, such as food composition and water activity. Mycotoxin production on carbohydrate-rich media has been studied extensively. However, very little is known of the effect of meat-based substrates on mycotoxin production by molds isolated from meat products. Some secondary metabolites derive from amino acids, including rugulovasine and cyclopiazonic acid from tryptophan *(20)*; verrucofortine from tryptophan and leucine *(19)*; cyclopenin, cyclopenol, viridicatin, and viridicatol from phenylalanine and methionine *(26)*; and anacine from leucine and glutamine *(23).* Different species of *Penicillium* produced verrucosidin and cyclopiazonic acid on a meat extract–based medium *(34, 42)* and citrinin and cyclopiazonic acid and ochratoxin A in meat products *(3, 14).* However, neither patulin nor ochratoxin A was detected after experimental inoculation of the producing molds on dry-cured ham *(3).*

The aim of the present work was to identify secondary metabolites produced by terverticillate penicillia in carbohydrate- and meat-based substrates as a means of characterizing the molds and to optimize an analytical technique based on the combination of high-pressure liquid chromatography (HPLC) coupled with photodiode array detection (DAD) and mass spectrometry (MS) to characterize terverticillate penicillia growing on dry-cured meat products.

MATERIALS AND METHODS

Identification of mold strains. The strains used had been collected from dry-cured hams and originally classified as terverticillate penicillia (*Penicillium* subgenus *Penicillium*) *(35).*

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Twenty one isolates from different stages of processing were selected. These isolates were identified by morphological characteristics on malt extract agar (MEA) *(36)* and Czapek yeast autolysate agar (CYA) *(36)* and by color reaction in four media containing sucrose and creatine at acidic or neutral pH: creatine sucrose agar (CREA), creatine sucrose dichloran agar (CREDA), creatine sucrose neutral agar (CSN), and creatine sucrose acidic agar (CSA) *(36–38).*

Cultivation of fungal strains. The selected strains were grown on six different substrates with different nutrient sources. Rice *(4)* and rice extract agar (REA) *(12)* were selected as media rich in complex carbohydrates. MEA is rich in dextrin and maltose. CYA was chosen as a sucrose-rich medium. Meat extract–triolein salt (MTS; containing 195 g/liter meat extract, 350 ml/liter triolein, 40 g/liter NaCl, and 0.1 g/liter $NaNO_3$) agar has a low carbohydrate content but is rich in both nitrogen compounds and lipids. Dry-cured ham slices were used as the model food for the studied molds.

Conidia from each selected mold were harvested by washing the surface of incubated MEA with sterile water containing 0.1% Tween 20. The resulting conidial suspensions were three-point inoculated in five petri plates each of MEA, CYA, REA, and MTS media. Ham slices in petri dishes and flasks containing 50 g of rice and 30 ml of distilled water were inoculated by spreading 1 ml of conidia suspensions.

MEA, CYA, REA, rice, and MTS cultures were incubated at 25°C for 14 days. Ham slices were kept at 25°C for 20 days, when sporulation was visible. Samples were collected at the end of the incubation period.

Preparation of fungal extracts. The contents of five petri dishes each of MEA, CYA, and REA were transferred to a plastic bag and macerated with 200 ml of chloroform in a Stomacher lab blender (model 400, Seward Medical, London, UK) for 4 min. After 1 h of incubation, the slurry was filtered twice through anhydrous sodium sulfate and Whatman no. 1 filter paper (Whatman International, Maidstone, UK). The filtrate was evaporated in a rotary evaporator (model VV2000, Heidolph, Kelheim, Germany) at 40°C, and the residue was resuspended in 5 ml of chloroform, filtered through a 0.45-μm-pore-size nylon membrane (MSI, Westboro, Mass.), and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4°C in the dark until required and resuspended in 200 μl of acetonitrile just before HPLC analysis.

To obtain the extracts from cultures on rice, 200 ml of chloroform was added and ultrasonicated for 30 min (Sonifier 250, Branson, Danbury, Conn.). After 1 h, the resulting mixture was filtered and handled as described above.

To prevent interference from the high fat content, a different procedure was followed to obtain the extracts from ham slices and MTS. After adding 5 ml of chloroform, the surface was scraped with a sterile glass stick. The suspension of conidia and mycelia was recovered with sterile Pasteur pipettes, ultrasonicated for 30 min, and extracted for 1 h. The extract was prepared in the same way as described for the other culture extracts.

Toxicity of fungal extracts. For the toxicity assays, only the extracts from MEA were used. The toxicity was evaluated with the brine shrimp test, and the cytotoxicity was evaluated with the neutral red *(5)* and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) *(39)* tests on VERO cells.

The brine shrimp test was performed as described by El-Banna et al. *(10)* with 200, 20, and 2 μg of extract per ml of brine shrimp medium *(18).* Dead larvae were counted just before incubation and after 24 and 48 h at 30°C in a shaker at 80 rpm. Toxicity of extracts was rated as follows: nontoxic, death rate of larvae not significantly different ($P < 0.05$) from spontaneous mortality in controls; slightly toxic, ≤49% mortality; toxic, 50 to 89% mortality; very toxic, ≥90% mortality.

The cytotoxicity assay was tested with the neutral red and MTT tests on VERO cells (ICN Biomedicals Ltd., Thame, UK). Cells were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Gaithersburg, Md.), supplemented with 10% fetal calf serum (GIBCO BRL), 1% penicillin-streptomycin solution (GIBCO BRL), and a 1% solution of 200 mM L-glutamine (GIBCO BRL). To obtain 2,000, 200, 20, and 2 μg of extract per ml of DMEM, appropriate volumes of extract were evaporated to dryness, dissolved in 25 μl of dimethylsulfoxide, and mixed with 1,200 μl of DMEM. To remove insoluble material, every mix was vigorously shaken and then centrifuged (7,200 × *g* for 5 min). Thereafter, 200 μl of the supernatant was incubated with 20,000 cells in individual wells of flat-bottom microtiter plates for 24 h at 37°C and 5% CO_2. Every dilution of extract was tested in triplicate. Viable cells were quantified after 3 h of incubation with neutral red and MTT dyes. Extracts were rated as nontoxic (no reduction in the average absorbance compared with that of controls), slightly toxic (≤49% reduction), toxic (50 to 89% reduction), and very toxic (≥90% reduction).

Analysis of secondary metabolites. Secondary metabolites in extracts obtained from different substrates were analyzed by HPLC-DAD and HPLC-MS. Secondary metabolites were identified according to their retention time, UV spectrum (190 to 390 nm), and molecular mass by comparison with reference values *(33).* Verrucosidin and cyclopiazonic acid were identified by comparison with standards. The verrucosidin standard was kindly supplied by Dr. L. Leistner (Institute for Microbiology, Toxicology and Histology, Kulmbach, Germany), and the cyclopiazonic acid was obtained from Sigma-Aldrich (St. Louis, Mo.). HPLC-DAD was carried out in a liquid chromatograph equipped with two pumps (model 126, Beckman, Palo Alto, Calif.) and a photodiode array detector (model 168, Beckman). HPLC-MS was performed in an HP series 1100 apparatus (Hewlett Packard, Palo Alto, Calif.) coupled with an LCQ mass spectrometer (Finnigan, San Jose, Calif.) with an atmospheric pressure chemical ionization (APCI) source. Positive ions were detected under the following conditions: vaporizer temperature of 450°C, sheath gas flow rate of 60 arbitrary units, auxiliary gas flow rate of 20 arbitrary units, discharge current of 5 μA, capillary temperature of 150°C, capillary voltage of 0 V, and tube lens offset of 0 V. For both HPLC-DAD and HPLC-MS, 5 μl of each extract was analyzed on a Supelcosil LC-18 column (25 cm long, 4.6 mm inside diameter, and 5 μm particle size; SUPELCO, Bellafonte, Pa.). Mobile phases were 100% water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). To achieve separation of metabolites, the flow rate was set at 0.8 ml/min and the following gradient was performed: initial 10% B for 0.5 min, linear change to 70% B in 20 min, 70% B for 10 min, linear change to 90% B in 2 min, 90% B for 5 min, linear change to 99% B in 1 min, 99% B for 10 min, linear change to 10% B in 2 min. The column was equilibrated at 10% B for 5 min.

RESULTS

Characterization of isolates. Only minor differences in macroscopic characteristics were found among the 21 selected isolates. Based on morphological characteristics and reactions in the creatine sucrose media, the 21 terverticillate penicillia isolates were classified into five groups

TABLE 1. *Differential morphological and physiological characteristics of five groups of selected terverticillate penicillia isolates*

Characteristic	Groups: A	B	C	D	E
Isolate code	13, 14	3, 16, 20	1, 2, 17	4, 7, 9, 10, 11, 12	21, 22, 23, 24, 25, 26, 27
MEA (25°C, 7 days)					
Colony diam (cm)	2.1–2.7	2.6–3.8	3.1–3.7	2.0–2.7	2.0–2.6
Colony aspect	Radially sulcate	Plane	Plane	Radially sulcate	Radially sulcate
Exudate	Absent	Absent	Absent	Brown	Absent
Soluble pigment	No	No	Yellow	No	No
CYA (25°C, 7 days)					
Colony diam (cm)	1.6–2.4	2.2–3.1	2.7–3.3	1.4–2.3	1.6–2.0
Exudate	Pale yellow	Pale yellow	Absent	Absent	None or colorless
Reverse	Pale yellow	Yellow brown	Yellowish orange	Orange	Yellow
CYA (37°C, 7 days)					
Colony diam (cm)	0	0	0.4	0	0
CREDA, CREA					
Exudate	Mauve	No	No	No	No
Soluble pigment	No	No	Orange	No	No
Medium reaction	Weakly acid	Weakly acid	Acid	Weakly acid	Weakly acid
Colony reverse	Alkaline	Alkaline	Acid	Alkaline	Alkaline
CSN, CSA					
Exudate	Mauve	None or mauve	Mauve	None or mauve	None or mauve
Soluble pigment	No	No	Orange	No	No
Medium reaction	Neutral	Neutral	Acid	Neutral	Weakly acid
Colony reverse	Weakly alkaline	Alkaline	Acid	Weakly alkaline	Weakly alkaline
Conidium wall	Smooth	Spinose	Smooth	Smooth	Smooth
Tentative identification	*P. commune, P. palitans*, or *P. solitum*	*P. echinulatum* or *P. discolor*	*P. polonicum* or *P. aurantiogriseum*	*P. commune, P. palitans*, or *P. solitum*	*P. commune, P. palitans*, or *P. solitum*

(Table 1). Isolates 3, 16, and 20 had spinose conidia and were tentatively characterized as *Penicillium echinulatum* or *Penicillium discolor* (group B). Isolates 1, 2, and 17 had a yellow or orange soluble pigment in most media and acid production on creatine-containing media and were tentatively characterized as *Penicillium polonicum* or *Penicillium aurantiogriseum* (group C). The remaining isolates had similar characteristics common to *Penicillium commune, Penicillium palitans,* and *Penicillium solitum.* Isolates 13 and 14 had similar exudates on the different media used and were classified as group A. Isolates 4, 7, 9, 10, 11, and 12 had a neutral reaction on CSN and CSA and were classified as group D, and the seven remaining isolates had a weak acid reaction and were included in group E.

Toxicity. The fungal extracts obtained from the five selected strains grown only on MEA were cytotoxic to VERO cells at the highest concentration of 2 mg/ml (Table 2). Three extracts showed cytotoxicity at the concentration of 0.2 mg/ml, but this result was restricted to the NR test. At this concentration, the toxicity against brine shrimp was high only for groups C and E. However, no toxic effect was observed at either of the lower concentrations tested (0.02 and 0.002 mg/ml) in any of the toxicity assays.

TABLE 2. *Toxicity in neutral red (NR), MTT, and brine shrimp tests of three concentrations of fungal extracts obtained from MEA*[a]

Group	0.02 mg/ml NR	MTT	Shrimp 24 h	Shrimp 48 h	0.2 mg/ml NR	MTT	Shrimp 24 h	Shrimp 48 h	2 mg/ml NR	MTT	Shrimp
A (*P. commune*)	−	−	−	−	+	−	−	−	+++	+++	ND
B (*P. echinulatum*)	−	−	−	−	−	−	−	−	++	++	ND
C (*P. polonicum*)	−	−	−	−	−	−	+++	+++	++	++	ND
D (*P. solitum*)	−	−	−	−	++	−	−	+	++	++	ND
E (*P. solitum*)	−	−	−	−	++	−	+++	+++	+++	+++	ND

[a] Results of cytotoxicity tests: +++, very toxic; ++, toxic; +, slightly toxic; −, nontoxic; ND, not determined.

TABLE 3. *UV absorption and positive ion mass of identified secondary metabolites*

Metabolite	UV absorption (nm)[a]	Monoisotopic ion mass
Rugulovasine A	**220**, 285, 288	269.1
Cyclopenol	**200**, 216, 278	311.1
Anacine		343.0
Viridicatol	**194, 221**, 241s, 286, 306, 318, 328	254.3
Cyclopenin	196, **212**, 232s, 251s, 290	295.1
Cyclopeptin	**214**, 232s, 252s, 292	281.0
Dehydrocyclopeptin	**193**, 210, 249s, 294s	279.2
Viridicatin	**192**, 205, **221**, 240s, 285, 307, 319, 331	238.0
3-*O*-Methylviridicatin	**192**, 204, **222**, 240, 288, 306, 318, 330	252.3
Verrucofortine	**205**, 248, 272, 285s	410.2
Puberulin	**198**, 214s, 248, 276, 280s	444.1
Arisugacin	**207**, 252, 331	471.1
Normethylverrucosidin	**204**, 240, 285	402.9
Cyclopiazonic acid	196, **224**, 280	337.1
Verrucosidin	**199**, 214s, 239, 295	416.9
Compactin	230s, **236**, 246s	391

[a] Numbers in bold indicate the highest absorption trace; s, shoulder.

Secondary metabolites. All the isolates were clustered into five groups according to their morphological characteristics. Only one isolate from each group was selected to further investigate the production of secondary metabolites. The five selected isolates of terverticillate penicillia produced a wide range of secondary metabolites on the various substrates. HPLC analysis of the chloroform extracts revealed a total of 16 compounds that were identified according to their UV spectra and molecular ion masses (Table 3).

Among the secondary metabolites produced by the selected isolate from group A (Fig. 1), rugulovasine and cyclopiazonic acid were identified (Table 4). Rugulovasine was detected in all the media except MTS, and cyclopiazonic acid was produced in every medium tested.

Seven secondary metabolites, including several viridicatols and arisugacin, were identified (Fig. 2) in extracts from the media inoculated with the selected isolate of group B (Table 5). All seven compounds were produced on MEA, REA, and rice. Most of these extrolites, including cyclopenol, cyclopenin, cyclopeptin, and arisugacin, were identified on CYA and ham slices. However, arisugacin but no viridicatol-related compound was detected on MTS.

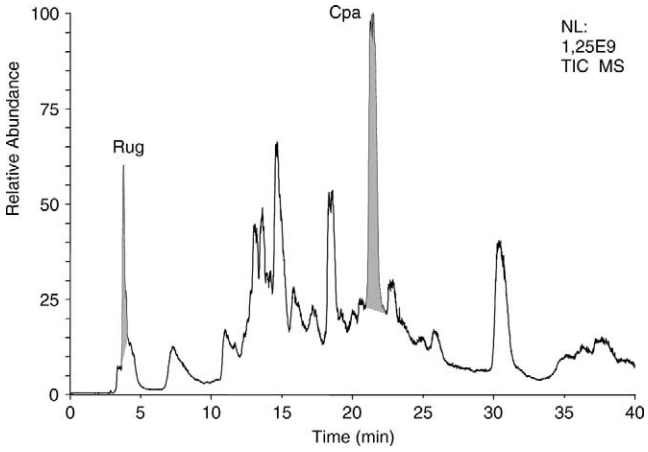

FIGURE 1. *HPLC-MS chromatograms of extracts from malt extract agar inoculated with a* Penicillium *isolate from group A. Identified metabolites: Rug, rugulovasine A; Cpa, cyclopiazonic acid.*

Eleven secondary metabolites, including viridicatols, anacine, puberulins, and verrucosidins, were identified in extracts from most media inoculated with the strain from group C (Fig. 3; Table 6). Only MTS did not support the production of detectable amounts of cyclopeptin, dehydrocyclopeptin, and viridicatin.

Groups D and E produced identical chromatographic patterns with seven secondary metabolites identified (Fig. 4), including viridicatols and compactin (Tables 7 and 8). Again, most of the substrates supported the synthesis of all these compounds, but MTS supported the production of only cyclopenol, cyclopenin, and cyclopeptin by the isolate from group D.

DISCUSSION

The mold species evaluated in this study are commonly found in dry-cured meat products *(1, 25, 35).* The highest concentration of the chloroform extract tested from the selected strains grown on MEA had toxigenic potential, but a concentration of 0.02 mg/ml revealed no toxic effect (Table 2). Because secondary metabolites of toxicological significance were produced at lower concentrations on ham slices than on MEA (Tables 4 through 8), the potential toxicity associated with the presence of the studied *Penicillium* species on meat products, such as dry-cured ham, is expected to be very low. Similar results have been obtained with the same toxicological tests from different strains isolated from dry-cured ham *(35).* For a better characterization of these molds, the production of secondary metabolites was evaluated by HPLC-DAD and HPLC-MS. As a result,

TABLE 4. *Secondary metabolites identified from different substrates inoculated with an isolate from group A (*Penicillium commune*)*[a]

Metabolite	HPLC-DAD		HPLC-MS					
	MEA	CYA	MEA	REA	Rice	CYA	Ham slices	MTS
Rugulovasine A	−	+	202	4,859	5,514	6,226	560	−
Cyclopiazonic acid	+	+	5,644	81	33	6,457	821	102

[a] Data are given as ion mass count in arbitrary area units per 10^6. +, detected; −, not detected.

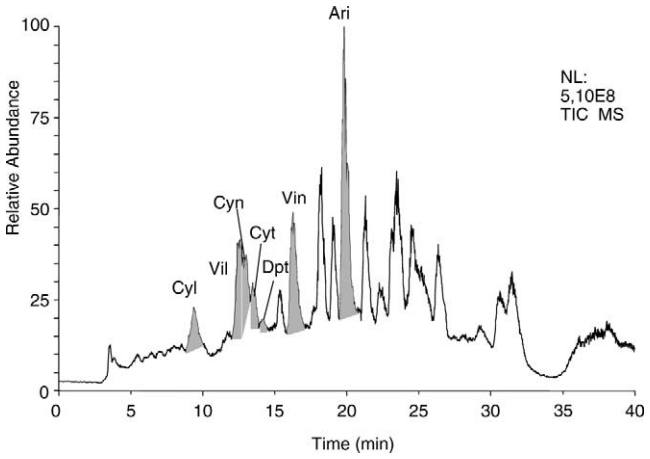

FIGURE 2. *HPLC-MS chromatograms of extracts from malt extract agar inoculated with a* Penicillium *isolate from group B. Identified metabolites: Cyl, cyclopenol; Vil, viridicatol; Cyn, cyclopenin; Cyt, cyclopeptin; Dpt, dehydrocyclopeptin; Vin, viridicatin; Ari, arisugacin.*

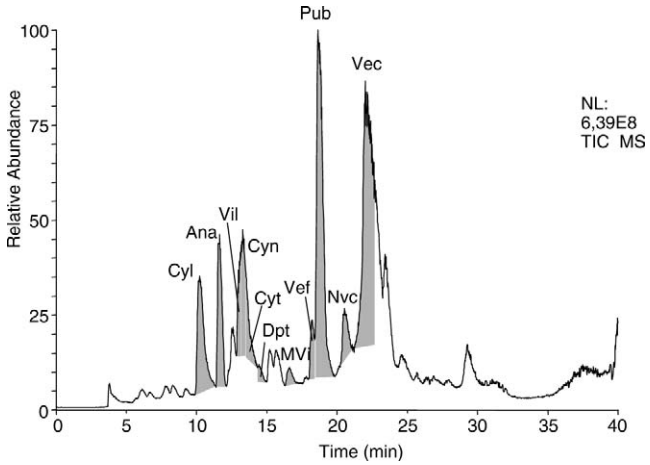

FIGURE 3. *HPLC-MS chromatograms of extracts from malt extract agar inoculated with a* Penicillium *isolate from group C. Identified metabolites: Cyl, cyclopenol; Ana, anacine; Vil, viridicatol; Cyn, cyclopenin; Cyt, cyclopeptin; Dpt, dehydrocyclopeptin; MVi, 3-*O*-methylviridicatin; Vef, verrucofortine; Pub, puberulin; Nvc, normethylverrucosidin; Vec, verrucosidin.*

several compounds were identified in the chloroform extracts from the different substrates used for each selected isolate (Tables 4 through 8). Analysis of profiles of secondary metabolites can lead to a more reliable identification of the mold strain studied because terverticillate penicillia are difficult to distinguish morphologically *(15).*

All the identified compounds have been reported in the literature as being produced by the tested species. Rugulovasine and cyclopiazonic acid, produced by group A, are typically produced by *P. commune (1, 15, 27, 42).* Because of this, the isolates in group A can be neither *P. solitum,* which synthesizes compactin, nor *P. palitans,* which does not produce rugulovasine *(15, 17, 27).*

Cyclopenol, viridicatol, cyclopenin, cyclopeptin, viridicatin, and dehydrocyclopeptin were produced by isolates of groups B, C, D, and E (Tables 5 through 8). These biosynthetically related viridicatols are very common metabolites of terverticillate *Penicillium* species such as *P. echinulatum, P. polonicum, P. solitum, P. aurantiogriseum, P. discolor, P. palitans, P. crustosum, P. hirsutum,* and *P. vulpinum (17, 41).*

In addition to viridicatols, group B isolates produced arisugacins, formerly named penechins, which are specific to *P. echinulatum (15, 17).* Similarly, group C isolates produced viridicatols, anacine, puberuline, and verrucosidin, which are specific to *P. polonicum (15, 17, 34).* The pattern of secondary metabolite production allows differentiation of *P. polonicum* from closely related *Penicillium* species *(28)* such as *P. aurantiogriseum, P. cyclopium,* and *P. viridicatum.*

Isolates in groups D and E produced compactin and viridicatols and thus were identified as *P. solitum (15, 17, 27).*

The ability of some of these molds to produce some of these metabolites in vitro should be considered one of the factors in risk assessment protocols because of their potential toxicity. Verrucosidin is a neurotoxin and is considered the most potent cytotoxin of the tremorgenic mycotoxins, with both genotoxic and mutagenic potential *(40).* Cyclopiazonic acid has been linked to Kodo poisoning in humans *(2),* has immunosuppressive activity at low doses, and causes necrosis of different tissues *(9).* Other metabolites have pharmacological effects: rugulovasine is a hypotensive alkaloid *(32),* arisugacins are acetylcholinesterase inhibitors *(22),* and compactin is an antihypercholesterolemic agent that affects DNA replication *(31).* However, high levels of compactin, also called mevastatin, are cytotoxic for acute myeloid leukemia cells *(43).* The remaining

TABLE 5. *Secondary metabolites identified from different substrates inoculated with an isolate from group B (*Penicillium echinulatum*)*[a]

	HPLC-DAD		HPLC-MS					
Metabolite	MEA	CYA	MEA	REA	Rice	CYA	Ham slices	MTS
Cyclopenol	+	+	242	5,566	22	309	22	−
Viridicatol	+	+	624	69	7	−	−	−
Cyclopenin	+	+	1,449	10,742	350	3,258	15	−
Cyclopeptin	−	−	139	6,481	994	87	5	−
Dehydrocyclopeptin	−	−	37	257	143	24	−	−
Viridicatin	+	−	1,932	2,140	113	−	−	−
Arisugacin	+	+	4,203	4,042	2,761	18,333	104	3,253

[a] Data are given as ion mass count in arbitrary area units per 10^6. +, detected; −, not detected.

TABLE 6. *Secondary metabolites identified from different substrates inoculated with an isolate from group C (*Penicillium polonicum*)*[a]

Metabolite	HPLC-DAD		HPLC-MS					
	MEA	CYA	MEA	REA	Rice	CYA	Ham slices	MTS
Cyclopenol	+	+	2,668	1,471	37	1,035	557	402
Anacine	−	−	3,179	473	232	227	1,690	187
Viridicatol	+	+	3,507	81	4	582	72	17
Cyclopenin	+	+	2,185	1,573	62	1,086	392	160
Cyclopeptin	+	−	744	206	524	95	129	−
Dehydrocyclopeptin	+	−	461	72	80	21	93	−
3-*O*-Methylviridicatin	+	+	591	226	12	408	108	−
Verrucofortine	+	+	1,922	15	266	537	955	215
Puberulin	+	+	11,371	988	2,883	3,331	6,359	1,713
Normethylverrucosidin	+	+	1,103	40	35	13	158	180
Verrucosidin	+	+	5,239	1,197	62	287	1,123	506

[a] Data are given as ion mass count in arbitrary area units per 10^6. +, detected; −, not detected.

compounds cannot be considered mycotoxins, but viridicatols can be toxic in a mixture with some other fungal metabolites *(16, 24).* Every studied strain produced bioactive compounds on MEA that can be responsible for the toxic effects observed. The cytotoxicity observed in extracts at 0.2 mg/ml can be due to cyclopiazonic acid from *P. commune* or compactin from *P. solitum.* Similarly, the toxicity obtained against brine shrimp larvae can be explained by verrucosidin and normethylverrucosidin from *P. polonicum* and by the higher levels of compactin and viridicatols from group E, identified as *P. solitum.* The low toxicity of arisugacins seems to explain the lack of effect of the 0.2 mg/ml extract from *P. echinulatum.* However, the high concentrations of both arisugacins and the remaining extrolites can explain the cytotoxic effect obtained with 2 mg/ml concentrations of the various extracts.

The production of secondary metabolites depends on substrate composition *(21, 34).* The carbohydrate-rich substrates used in this study (rice, REA, MEA, and CYA) support the production of most of secondary metabolites by penicillia isolated from dry-cured ham (Tables 4 through 8). All the secondary metabolites identified from every organism tested were obtained in the substrates containing complex carbohydrates, i.e., rice and REA. The sucrose-rich medium CYA also supported the production of most mycotoxins at levels similar to those obtained on MEA, except that *P. echinulatum* did not produce detectable amounts of viridicatol and viridicatin on this particular culture medium. These compounds can be converted from cyclopenin and cyclopenol by a single enzymatic step *(26).* The same isolate of *P. echinulatum* could produce viridicatin and viridicatol in the other substrates; therefore, there must be an inhibitory effect in this enzymatic step.

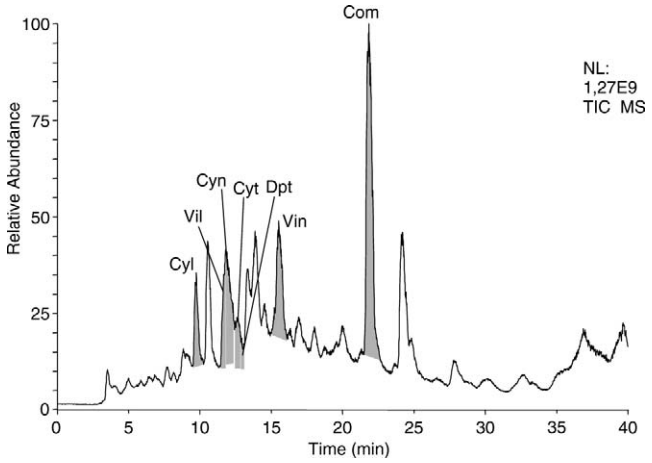

FIGURE 4. *HPLC-MS chromatograms of extracts from malt extract agar inoculated with a* Penicillium *isolate from group D. Identified metabolites: Cyl, cyclopenol; Vil, viridicatol; Cyn, cyclopenin; Cyt, cyclopeptin; Dpt, dehydrocyclopeptin; Vin, viridicatin; Com, compactin.*

Dry-cured ham slices, which are low in carbohydrates, also supported the synthesis of most secondary metabolites by each *Penicillium* species (Tables 4 through 8), a fact that has not been reported previously. As with CYA and *P. echinulatum,* some viridicatols were not detected in the extracts from ham slices. The high level of amino acids in dry-cured ham *(8)* could increase the synthesis of nitrogen-containing secondary metabolites. Although the efficiency of the methods used to recover the metabolites differs for the various substrates, the production of extrolites in dry-cured ham does not reach levels as high as those in carbohydrates-rich substrates. In this work the metabolites were extracted from fungal mycelia and not from the ham. The results obtained with MTS revealed that several metabolites were not detected, including viridicatols from *P. echinulatum,* some viridicatols from *P. solitum* and *P. polonicum,* and rugulovasine from *P. commune.* Thus, higher amino acid content does not imply the production of higher levels of extrolites. Individual components of the media or a combination of these components may have an inhibitory effect. In addition, some of the metabolites may react with other components of the food, modifying its chromatographic properties and its UV and MS characteristics and remaining unnoticed.

The studied terverticillate *Penicillium* species are capable of producing a number of secondary metabolites in a wide range of substrates, including dry-cured ham, under optimal growth conditions. This potential should be carefully evaluated when assessing the risk posed by the pres-

TABLE 7. *Secondary metabolites identified from different substrates inoculated with an isolate from group D (*Penicillium solitum*)*[a]

	HPLC-DAD		HPLC-MS					
Metabolite	MEA	CYA	MEA	REA	Rice	CYA	Ham slices	MTS
Cyclopenol	+	+	528	1,996	44	2,118	322	16
Viridicatol	+	+	275	132	185	273	203	−
Cyclopenin	+	+	734	2,954	196	2,542	141	30
Cyclopeptin	+	+	92	1,751	1,869	151	331	11
Dehydrocyclopeptin	+	+	20	244	263	24	225	−
Viridicatin	−	+	845	961	2,471	207	106	−
Compactin	+	+	1,843	26	225	1,588	20	−

[a] Data are given as ion mass count in arbitrary area units per 10^6. +, detected; −, not detected.

ence of these species on dry-cured meats. During the different stages of production of ham, environmental conditions and microbiological composition vary significantly *(8, 35)* and may be unfavorable for the synthesis of secondary metabolites. Contrary to the design of this study, individual molds do not grow as isolated entities on cured ham in production facilities but rather grow in competition with each other and with bacteria. This interaction may reduce the production of metabolites or these compounds might be metabolized by other microorganisms. Hazardous extrolites can be excreted to the meat product, as it has been shown for ochratoxin A, citrinin, and cyclopiazonic acid *(3, 13, 14).* Some mycotoxins rapidly decrease after direct contamination of dry-cured ham, but cyclopiazonic acid concentration remained at more than 80% of the initial concentration during processing and storage *(3).* Some amino acids are capable of reacting with particular mycotoxins such as penicillic acid to form abducts of a lower toxicity *(7).* The inferred risk would be overestimated given that the mycelium is usually brushed off of the ham surface before wholesale distribution. As a consequence, the risk due to these metabolites on dry-cured meat products and other mold-ripened foods should be better characterized and the actual levels of these compounds present in such foods should be studied. We found that HPLC in combination with DAD and MS is a suitable analytical technique for this purpose. This technique can be used to monitor the presence of these fungal species during the production of ham, which would be difficult if identification were based on only morphological characteristics. Selected nontoxigenic molds can be used as starter cultures to prevent the occurrence of hazardous metabolites while keeping the beneficial contribution of fungi to the ripening of dry-cured meat products.

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TABLE 8. *Secondary metabolites identified from different substrates inoculated with an isolate from group E (*Penicillium solitum*)*[a]

	HPLC-DAD		HPLC-MS					
Metabolite	MEA	CYA	MEA	REA	Rice	CYA	Ham slices	MTS
Cyclopenol	+	+	71	323	20	12	105	−
Viridicatol	+	+	436	40	25	−	29	−
Cyclopenin	+	+	2,169	5,321	89	745	113	−
Cyclopeptin	+	+	326	820	2,669	131	74	−
Dehydrocyclopeptin	+	+	37	106	285	15	−	−
Viridicatin	+	+	2,329	633	784	86	−	−
Compactin	+	+	6,116	494	117	5,392	−	−

[a] Data are given as ion mass count in arbitrary area units per 10^6. +, detected; −, not detected.

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