

***Penicillium expansum*: Consistent Production of Patulin,  
 Chaetoglobosins, and Other Secondary Metabolites in Culture  
 and Their Natural Occurrence in Fruit Products**

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*Penicillium expansum* is known for its destructive rot and patulin production in apple juice. According to the literature, *P. expansum* can, among other compounds, produce citrinin, ochratoxin A, patulin, penitrem A, and rubratoxin B. In this study the qualitative production of metabolites was examined using TLC (260 isolates), HPLC (85 isolates), and MS (22 isolates). The results showed that none of the 260 isolates produced ochratoxin A, penitrem A, or rubratoxin B. However, chaetoglobosin A and communesin B were produced consistently by all 260 isolates. Patulin and roquefortine C were produced by 98% of the isolates. Expansolides A/B and citrinin were detected in 91 and 85% of the isolates, respectively. Chaetoglobosins and communesins were detected in naturally infected juices and potato pulp, whereas neither patulin nor citrinin was found. Because most *P. expansum* isolates produce patulin, citrinin, chaetoglobosins, communesins, roquefortine C, and expansolides A and B, foods contaminated with this fungus should ideally be examined for chaetoglobosin A as well as patulin.

**KEYWORDS:** Apple juice; cherry juice; metabolite profiling; mycotoxins; qualitative detection methods

**INTRODUCTION**

*Penicillium expansum* is one of the most common foodborne fungi on fruit. It typifies the genus *Penicillium* and is therefore also one of the most studied species in the genus (1). *P. expansum* is often found on rotting apples, pears, and cherries, but it is also common on walnuts, pecans, hazelnuts, and acorns (2). *P. expansum* is of concern especially in fruit products because of its production of patulin. Specific regulation for patulin at a level of 50 µg/kg has been set by most countries in Europe, and several quantitative methods have been developed (3, 4). However, these regulations and methods deal only with patulin, and *P. expansum* produces many other toxic metabolites.

In pure culture, *P. expansum* is reported to produce no fewer than 50 different secondary metabolites (5–13), such as cyclopiazonic acid (5), aflatrem (6), brevianamide A (5–7), chaetoglobosins A and C (5, 9, 10), citrinin (7, 11, 14), expansolides A and B (12), gentisyl alcohol (5), griseofulvin (5), mycophenolic acid (5), ochratoxin A (7), patulin (7, 11, 14), penicillic acid (14), penitrem A (5), PR-toxin (6), Raistrick phenols (10), roquefortine C (5, 11), and rubratoxin B (7), but recent studies suggest that the range of metabolites is much more limited (15, 16). Some of these metabolites have also been reported in foods. Patulin has been detected in fruits and juices (8, 17), and trace amounts of citrinin have been found in moldy apples and apple juice (18–20). Ciegler et al. (18) showed that

the combined toxicity of patulin and citrinin is additive and emphasized the need for examining strains of *P. expansum* for other toxic metabolites, as they could have additive or even synergistic effects.

To assess if other metabolites from *P. expansum* could constitute a health hazard to humans, it is necessary first to know which metabolites *P. expansum* is capable of producing in pure culture and if they are consistently produced. This is done by qualitative, multimetabolite analyses comparing many isolates from different habitats. Then the same qualitative, multimetabolite analyses are applied to fruits and fruit products naturally contaminated with *P. expansum* to establish if the same metabolites have been produced.

One purpose of this study was to demonstrate that the metabolite production of different *P. expansum* isolates in pure cultures is consistent regardless of origin. Another purpose was to show that *P. expansum*, when contaminating fruits and fruit products, can produce toxic metabolites other than patulin and to focus the attention of legislators and people in the food industry on the potential problem of co-occurring *P. expansum* metabolites in fruit products.

**MATERIALS AND METHODS**

**Morphological Identification.** The identities of several hundred *Penicillium* isolates from various international culture collections, which were claimed either to be *P. expansum* or to produce patulin or chaetoglobosins, were checked. They were inoculated onto Czapek yeast

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extract agar (CYA) (21) and malt extract agar [(MEA), according to the method of Blakeslee (21)] and rigorously examined using micro-morphological and macromorphological characters according to the method of Pitt (1). Together with isolates in the IBT collection, 260 typical *P. expansum* isolates were selected for this study.

**Media, Growth Conditions, and Experimental Setup.** All 260 isolates were inoculated onto CYA, MEA, potato dextrose agar (PDA, Difco), and yeast extract sucrose agar (YES) (21). One milliliter of trace metal solution (1.0 g of  $ZnSO_4 \cdot 7H_2O$  and 0.5 g of  $CuSO_4 \cdot 5H_2O$  per 100 mL) was added to all media according to the method of Filtenborg et al. (22). The inoculated plates were packed in perforated plastic bags and incubated at 25 °C in darkness for 7 days prior to examination. In the first part of this study, all 260 *P. expansum* isolates were studied. They were screened for their production of citrinin, communesin B, patulin, and roquefortine C using TLC. In the second part, 85 isolates representing as many different substrata and countries as possible were selected (Table 2). They were analyzed by HPLC-UV for the production of citrinin, chaetoglobosins, communesins, and patulin, while a randomly chosen subset of 22 isolates were also analyzed using direct injection ES-MS and LC-MS for the detection of expansolides A/B and confirmation of the above-mentioned metabolites. All fungal cultures are held at the IBT Culture Collection at BioCentrum-DTU, Denmark.

**TLC Analysis.** The 260 isolates were screened by TLC using the agar plug method of Filtenborg et al. (23, 24). Plugs (4 mm in diameter) from PDA, CYA, and YES were wetted with a drop of chloroform/methanol (2:1, v/v) and placed separately mycelium side down on silica gel 60 plates. The plates were eluted in two different eluent systems: TEF (toluene/ethyl acetate/90% formic acid, 5:4:1, v/v/v) and CAP (chloroform/acetone/2-propanol, 85:15:20, v/v/v). Three spray reagents were used to develop the plates: MBTH [1% 3-methylbenzothiazol-2-one-hydrazone hydrochloride in water (w/v)];  $H_2SO_4$  (6 N sulfuric acid/methanol, 1:1, v/v); and  $Ce(SO_4)_2$  [1%  $Ce(SO_4)_2$  in 6 N sulfuric acid (w/v)]. Griseofulvin was used as an external standard on each TLC plate, and all  $R_f$  values of the metabolites were calculated relative to the  $R_f$  of griseofulvin [ $R_f(\text{citrinin}) = R_f(\text{citrinin})/R_f(\text{griseofulvin})$ ]. The production of metabolites was confirmed by standards of citrinin, communesin B, patulin, and roquefortine C from the IBT Metabolite Collection.

**Extraction.** Five 6 mm agar plugs were cut from each of the 85 cultures of both the CYA and YES cultures and placed separately in 2 mL vials. One milliliter of ethyl acetate/formic acid (200:1, v/v) was added to each vial, and the plugs were extracted for 60 min by sonication. Each extract was transferred to a clean 2 mL vial and evaporated to dryness in a rotary vacuum concentrator. The dried residue was redissolved ultrasonically in 500  $\mu$ L of methanol for 10 min and filtered through a 0.45  $\mu$ m filter into a clean 2 mL vial prior to HPLC and MS analyses.

**Naturally Infected Samples.** Six samples of naturally infected fruits, juices, and pulps sampled from orchards, factories and a farm were also examined. The fungi were isolated, identified, and analyzed by HPLC and included in the 85 isolates. The samples were extracted with ethyl acetate/formic acid (100:1, v/v), evaporated to dryness, redissolved in 1 mL of methanol by sonication for 30 min, filtered through a 0.45  $\mu$ m filter into a clean 2 mL vial, and analyzed in the same HPLC system as extracts of the pure cultures.

**HPLC-UV Analysis.** This was performed on an HP-1100 high-performance liquid chromatograph equipped with a diode array detector (DAD) (Agilent, Waldbronn, Germany). Separations were done on a 125  $\times$  2 mm i.d., 3  $\mu$ m Hypersil BDS-C<sub>18</sub> cartridge column (Agilent, Palo Alto, CA) with a 10  $\times$  2 mm i.d. Superspher 100 RP-18 guard column (Agilent). A linear gradient started at 85% water and 15% acetonitrile, changing to 100% acetonitrile in 40 min and maintaining this for 5 min. The solvent composition was returned to starting conditions in 8 min followed by 5 min of equilibration. A flow rate of 0.3 mL/min was used. Both eluents contained 50  $\mu$ L/L (v/v) trifluoroacetic acid. The column temperature was 40 °C, and the injection volume was 3.0  $\mu$ L. All chemicals used were of Merck analytical grade, and the water was of Milli-Q grade (Waters). A homologous series of alkylphenones was analyzed as external retention time references and used to calculate a bracketed retention index (RI) (25).

Standards from the IBT Metabolite Collection of chaetoglobosins A and C, communesins A and B, expansolides A/B, patulin, roquefortine C, and citrinin were used to confirm the identity of individual peaks by comparing RI and UV spectra. Furthermore, using analytical reference data, all extracts were likewise screened for the presence of the following metabolites: 6-methylsalicylic acid,  $\alpha$ -cyclopiazonic acid, aflatoxin B<sub>1</sub>, aflatrem, alternariol, alternariol monomethylether, auran-tioclavine, brevianamide A, carlosic acid, chrysophanol, compactin, cyclophenol, erythroglauconin, frequentin, fulvic acid, gentisyl alcohol, griseofulvin, griseophenone C, islandicin, kojic acid, lapidosin, myco-phenolic acid, naphthalic anhydride, norlichexanthone, ochratoxin A, pachybasic acid, palitantin, penitrem A, PR-toxin, purpurogenone, roquefortine B, roquefortine D, rubratoxin B, terrein, terrestric acid, viomellein, xanthocillin X, and xanthomegnin. All standards are held at the IBT Metabolite Collection at BioCentrum-DTU, Denmark, and were previously analyzed under the same conditions (26).

**Direct Infusion ESI-MS Analysis.** A subset of 22 extracts were analyzed to confirm the identity of metabolites and detection of expansolides A/B, by direct infusion electrospray mass spectrometry (ESI-MS) as described by Smedsgaard and Frisvad (27, 28) or adapted to high-resolution accurate mass spectrometry: a 1  $\mu$ L aliquot of extract was infused into the ESI source at a rate of 15  $\mu$ L/min using methanol as carrier from an Agilent 1100 microflow pump. Just prior to the source, water with 2% formic acid was added from a Harvard syringe pump at a rate of 5  $\mu$ L/min, giving a combined flow of 75% methanol with 0.5% formic acid at a rate of 20  $\mu$ L/min. The quadrupole time-of-flight mass spectrometer, Q-tof (Micromass) was tuned to a resolution of better than 8500 fwhm and calibrated on a PEG solution to a residual error of <2 mDa. One scan per second was collected from  $m/z$  150 to 1000 as continuum spectra. About 10 scans were summarized and converted into a centroid using protonated roquefortine C (390.1930 Da) as internal mass reference.

**HPLC ESI-MS Analysis.** The production of metabolites in culture and that on naturally infested sample were compared by high-resolution LC-MS: 3  $\mu$ L of extract was injected into an Agilent 1100 system coupled to an LCT time-of-flight mass spectrometer by an electrospray ion source with a LockMass probe (Micromass). Separation was done on a 50 mm  $\times$  2 mm i.d. Luna C<sub>18</sub> (2) column (Phenomenex, Torrance, CA) at a flow rate of 0.4 mL/min by a linear water/acetonitrile gradient (both with 20 mM formic acid) changing from 10% acetonitrile in water to 100% acetonitrile in 20 min; 100% acetonitrile was held for 5 min before the gradient was returned to starting conditions. The instrument was tuned to a resolution of >6000 fwhm, calibrated on a solution of PEG, and a solution of leucineenkephaline (1  $\mu$ g/mL in 50% acetonitrile with 0.1% formic acid) was used as lockmass at a flow rate of 5  $\mu$ L/min. Two centroid mass measured scans were collected per second from  $m/z$  150 to 900.

## RESULTS

**Morphological Identification.** All 260 *Penicillium* isolates identified as *P. expansum* had smooth ellipsoidal conidia and smooth terverticillate stipes. Colonies on CYA exceeded 30 mm after 7 days of growth at 25 °C and had an orange-brown reverse. Colonies on MEA were fasciculate and had white margins.

**TLC Analysis.** Extracts of pure cultures of *P. expansum* and authentic standards of communesin B, citrinin, patulin, and roquefortine C were compared in two eluent systems with different selectivities. The results showed that all 260 *P. expansum* isolates produced the same basic profile of known and unknown metabolites. Apart from communesin B, citrinin, patulin, and roquefortine C, shown in Figure 1, three unknown metabolites with conspicuous color reactions were detected by TLC (Table 1). Communesin B and unknowns 1 and 3 were consistently detected in plugs from all 260 isolates of *P. expansum*. Roquefortine C was found in 255 of the isolates, and patulin and unknown 2 were found in 244, whereas citrinin was found in only 187 of the isolates. The presence of citrinin,

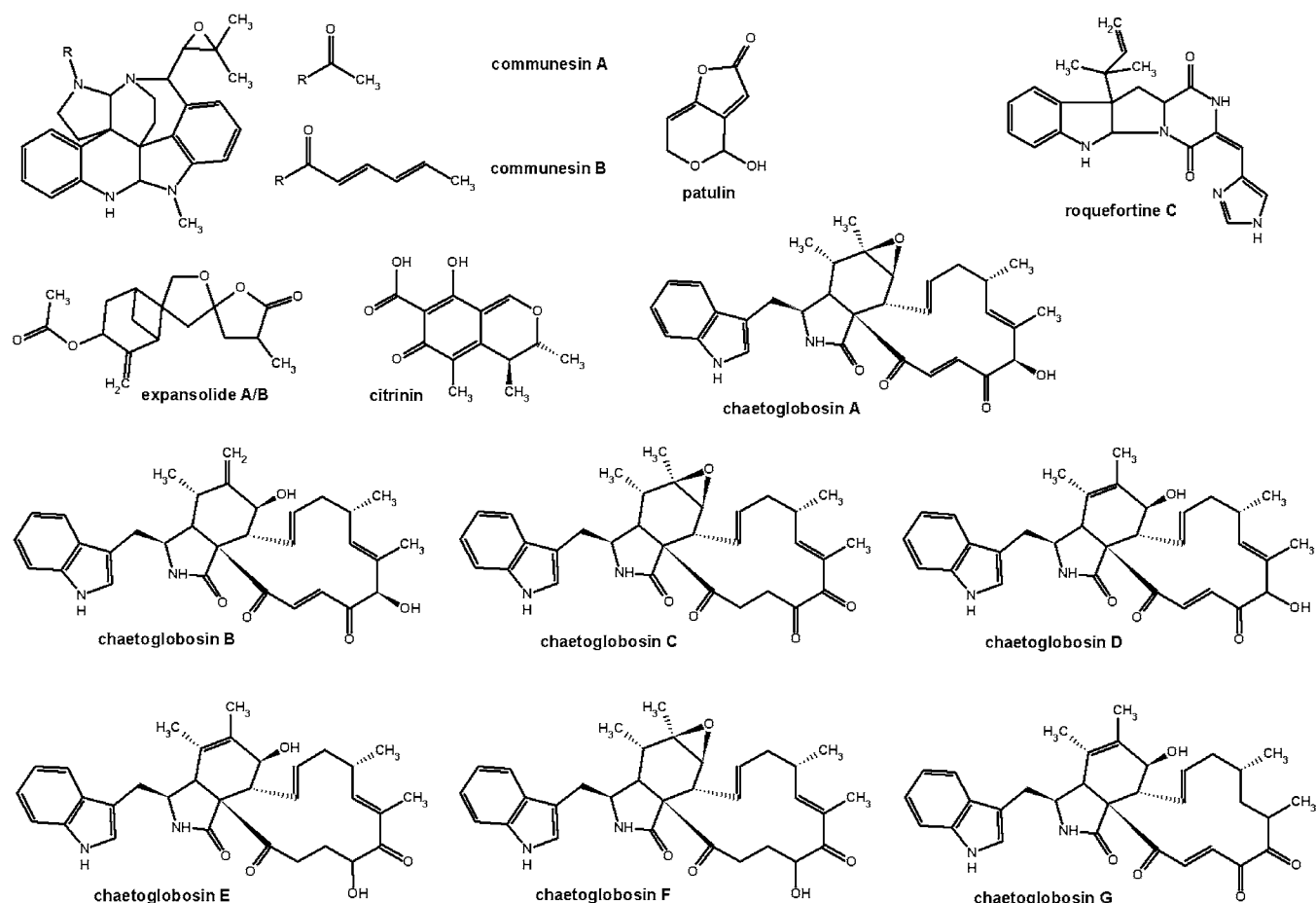


Figure 1. Structures of the metabolites produced by *P. expansum*.

Table 1.  $R_f$  Values Relative to Griseofulvin ( $R_{fg}$ ), Color from TLC, and RI Values from HPLC for Metabolites Produced by *P. expansum*

metabolite	TLC			HPLC
	$R_{fg}$ (CAP)	$R_{fg}$ (TEF)	treatment/UV 366-vis light (color)	RI
citrinin	0.18	1.16	none/UV 366 [yellow (tail in TEF)]	882
unknown 1	— <sup>a</sup>	1.18	none/UV 366 (fluorescent blue)	—
roquefortine C	0.52	0.09	Ce (SO <sub>4</sub> ) <sub>2</sub> /vis (orange)	1139
unknown 3	1.11	—	Ce (SO <sub>4</sub> ) <sub>2</sub> /UV 366 (fluorescent light blue)	—
patulin	0.90	1.00	MBTH/vis (yellow)	639
unknown 2	0.60	0.84	MBTH/vis (pink)	—
communesin B	1.04	0.25	Ce (SO <sub>4</sub> ) <sub>2</sub> + MBTH/vis (blue)	1344

<sup>a</sup> —,  $R_{fg}$  and RI values not known.

communesins B, patulin, and roquefortine C was confirmed with the standards.  $R_{fg}$  values and color reaction for the known metabolites together with three unknown metabolites are given in Table 1. None of the 260 *P. expansum* isolates analyzed by TLC in this study were found to produce any spots that had the same  $R_{fg}$  value and color as ochratoxin A [ $R_{fg}$  (CAP/TEF) = 0.23/1.51 with blue green fluorescence].

The TLC screening showed that all seven metabolites were produced on all three media but that the production of patulin and unknown 2 was higher in PDA cultures, whereas the production of communesin B, roquefortine C, and unknown 3 was higher in CYA cultures. Citrinin and unknown 1 were produced in larger amounts in YES cultures. The combined TEF system and treatment with H<sub>2</sub>SO<sub>4</sub> was the best detection method for citrinin and unknown 1, whereas CAP combined with Ce(SO<sub>4</sub>)<sub>2</sub> spray was best for the detection of communesin B, roquefortine C, and unknown 3. Furthermore, if the TLC plate treated in CAP/Ce(SO<sub>4</sub>)<sub>2</sub> was treated afterward with MBTH and

dried gently, communesin B would show up as a sky-blue spot in daylight. Patulin and unknown 2 were equally well detected in TEF and CAP when the TLC plate was treated with MBTH.

**HPLC-UV Analysis.** The chromatograms from HPLC-UV analyses of the 85 *P. expansum* isolates were very similar and showed that all isolates tested consistently produced chaetoglobosin A and communesin B, whereas chaetoglobosin C and several other chaetoglobosins were detected in 37 of the isolates and communesin A was detected in 40 of the isolates. Production of patulin and citrinin was found in 83 and 72, respectively, of the isolates. Table 2 summarizes the metabolite production of the isolates according to their substratum and origin. Furthermore, aurantioclavin and gentisyl alcohol were detected in ~50% of the extracts. Expansolides A/B cannot be detected by HPLC-UV, because it only has an end absorption below 205 nm. Several unknown metabolites also were detected in most of the 85 isolates. No peak in the 85 chromatograms of *P. expansum* analyzed in this study was found to have a retention

Table 2. Mycotoxins and Other Metabolites<sup>a</sup> Produced by 85 *P. expansum* Isolates According to Substratum Groups and Geographic Origin

substratum	origin	Cha	Cit	Com	Pat	Roq	isolate
fruits							
apple	Australia	+	+	+	+	+	FRR 1600
apple	Czech Republic	+	+	+	+	+	CCF 1637
apple	Czech Republic	+	+	+	ND	+	CCF 1638
apple	Denmark	+	+	+	+	+	IBT 3717; IBT 4085; IBT 5887; IBT 6096; IBT 6098; IBT 6102; IBT 6108; IBT 6243; BA 843–845; BA 849
apple	Denmark	+	ND	+	+	+	IBT 6105; IBT 15305
apple	England	+	+	+	+	+	IBT 15684
apple	Germany	+	+	+	+	+	IBT 3484
apple	Hungary	+	+	+	+	+	IBT 15622
apple	Sweden	+	+	+	+	+	IBT 15683
apple	USA	+	+	+	+	+	NRRL 976 <sup>b</sup>
apple	USA	+	ND	+	+	+	IBT 21026
apple pulp	Denmark	+	+	+	+	+	BA 1029
apricot	Denmark	+	+	+	+	+	IBT 4946
black currant	Denmark	+	+	+	+	+	IBT 10898
cherry	Denmark	+	+	+	+	+	BA 600; BA 607; BA 613
cherry juice	Denmark	+	+	+	+	+	IBT 15717
gooseberry juice	Denmark	+	+	+	+	+	IBT 15716
pear	Denmark	+	+	+	+	+	IBT 6107
pear puree	Australia	+	+	+	+	+	FRR 48
prune	Denmark	+	+	+	+	+	IBT 12927
quince	Bulgaria	+	+	+	+	+	IMI 174158
rowanberry	Germany	+	+	+	+	+	IBT 17204; IBT 17217
strawberry jam	Denmark	+	+	+	+	+	IBT 5868; IBT 10948
vegetables							
beetroot	Denmark	+	+	+	+	+	IMI 285521
beetroot	Denmark	+	ND	+	+	+	rka-44
Brussels sprout	Denmark	+	+	+	+	+	CBS 481.84
carrot	Denmark	+	+	+	+	+	IBT 5867
licorice root	Thailand	+	+	+	+	+	IBT 6078
tomato		+	+	+	+	+	BA 537
cereals							
corn	Slovenia	+	+	+	ND	+	IBT 16705
wheat	India	+	+	+	+	+	IMI 232297
nuts							
acorn	Denmark	+	+	+	+	+	IBT 11501; IBT 11634
acorn	Denmark	+	ND	+	+	+	IBT 11499
spruce cone	Denmark	+	+	+	+	+	IBT 13745
spruce cone	Denmark	+	ND	+	+	+	IBT 13746
walnut	Denmark	+	ND	+	+	+	IBT 6094
processed commodities							
chilled food	France	+	ND	+	+	+	IBT 15598
liver pâté	Denmark	+	+	+	+	+	IBT 3483
margarine	Australia	+	ND	+	+	+	FRR 4314
meat	Germany	+	+	+	+	+	CBS 481.75
salami	Germany	+	+	+	+	+	IBT 6090
salami	Germany	+	ND	+	+	+	IBT 6086
other							
air, indoor	Denmark	+	+	+	+	+	IBT 6249; IBT 22437
air, indoor	Denmark	+	ND	+	+	+	IBT 23015
salt lake	Israel	+	+	+	+	+	IBT 23912
soil	Germany	+	+	+	+	+	IBT 5886
soil	Norway	+	+	+	+	+	IBT 24299
soil	Norway	+	ND	+	+	+	IBT 24108
soil	USA	+	+	+	+	+	IBT 15585
soil, alkaline	Denmark	+	+	+	+	+	IBT 19300
	Canada	+	+	+	+	+	DAOM 215349; DAOM 315350
	Canada	+	ND	+	+	+	DAOM 215351
	Czech Republic	+	+	+	+	+	IBT 19545
	England	+	+	+	+	+	NRRL 2021 <sup>c</sup>
	Germany	+	+	+	+	+	CBS 110404
	Moldavia	+	+	+	+	+	VKM F-1971
	Russia	+	+	+	+	+	VKM F-276; VKM F-2545
	Turkey	+	+	+	+	+	IBT 15603; IBT 15626
	USA	+	+	+	+	+	IBT 21771; RMF 7831; RMF 7842
		+	+	+	+	+	IBT 3708; IBT 10897; IMI 277247

<sup>a</sup> Cha, chaetoglobosins; Cit, citrinin; Com, communesins; Pat, patulin; Roq, roquefortine C. <sup>b</sup> Culture ex neotype of *P. expansum*. <sup>c</sup> Culture ex type of *P. resticulosum*.

index (RI) and/or UV spectrum corresponding to that of ochratoxin A (RI = 1098), penitrem A (RI = 1283), rubratoxin B (RI = 989), or any of the other 37 standards examined (26).

**Direct Infusion ESI-MS Analysis.** Twenty-two extracts were selected for analyses by direct injection electrospray mass spectrometry (ESI-MS). Ions corresponding to the protonated

molecular mass indicated the presence of the major secondary metabolites produced by *P. expansum* as chaetoglobosins A–D and/or G at *m/z* 529, chaetoglobosins E and/or F at *m/z* 531, communesin A at *m/z* 457, communesin B at *m/z* 509, roquefortine C at *m/z* 390, expansolides A and/or B at *m/z* 307, and citrinin at *m/z* 251. The structures of these metabolites are

**Table 3.** Natural Occurrence of Mycotoxins and Other Metabolites of *P. expansum* in Foods and Feeds Analyzed by HPLC

moldy sample	Cit	Pat	Cha A	Cha C	Com B	Roq C
windfall apples	ND	+	+	+	+	+
apple on the tree	ND	ND	+	ND	ND	ND
apple pulp	ND	+	ND	ND	ND	ND
potato pulp	ND	ND	+	+	+	ND
cherry juice	ND	ND	+	+	+	+
gooseberry juice	ND	ND	+	ND	+	ND

shown in **Figure 1**. By using direct infusion high-resolution accurate mass spectrometry, the identity of these ions could be confirmed with an accuracy better than 5 ppm using roquefortine C as internal mass reference [typical results: citrinin measured 251.0938 Da, calculated 251.0909 Da (mass error 7.4 ppm); communisin A measured 457.2618 Da, calculated 457.2604 Da (mass error 3.2 ppm); communisin B measured 509.2914 Da, calculated 509.2917 Da (mass error -0.5 ppm); chaetoglobosin A-D/G measured 529.2698 Da, calculated 529.2702 Da (mass error -0.8 ppm); chaetoglobosin E/F measured 531.2868 Da, calculated 531.2859 Da (mass error 1.7 ppm)]. Furthermore, the chaetoglobosins and communisins could also be seen as sodiated molecular mass with the addition of 23 Da; therefore, an overlap between, for example, chaetoglobosin A ( $M + H^+$ ) at  $m/z$  531 and communisin B ( $M + Na^+$ ) at  $m/z$  531 has to be taken into account. An ion pattern corresponding to the protonated chaetoglobosins (A-D and G) was found in all samples. All 22 isolates consistently showed ions corresponding to roquefortine C and the communisins (A and B), whereas the presence of citrinin was verified in 19 of the 22 isolates, confirming the TLC results. Ions corresponding to expansolides A/B, which are detected by MS only, were found in 20 of the 22 tested isolates. Patulin was not detected by this ES-MS method. No ions corresponding to either the protonated or the sodiated ions of ochratoxin A (403 Da), penitrem A (633 Da), or rubratoxin B (518 Da) were found in any of the 22 *P. expansum* isolates analyzed by MS.

**Consistency and Natural Occurrence.** The results in **Table 2** show that most of the 85 *P. expansum* isolates, which were a representative selection of the initial 260 isolates, came from fruit, with apples as the overall dominating substratum. *P. expansum* was also found frequently on different types of berries and nuts, but seldom found on cereals. Furthermore, most of the 260 isolates came from the temperate climate zone and, regardless of origin and substratum, the profiles of metabolites produced were the same for the majority of *P. expansum* isolates.

Fungal analyses of the naturally infected samples showed that *P. expansum* grew as a monoculture on all samples except the apple pulp, in which it occurred with *Alternaria* species. The two *P. expansum* isolates (IBT 15717 and BA 849) originally isolated from cherry juice and windfall apples, respectively, produced chaetoglobosins A and C, communisins A and B, citrinin, patulin, and roquefortine C in pure cultures. The two *P. expansum* isolates from gooseberry juice and apple pulp (IBT 15716 and BA 1029, respectively) were able to produce chaetoglobosin A, communisin B, citrinin, patulin, and roquefortine C, whereas IBT 15305 from a moldy apple produced only chaetoglobosin A, communisin B, patulin, and roquefortine C and citrinin, chaetoglobosin C, or communisin A was not detected. *P. expansum*, IBT 3222, isolated from potato pulp, was no longer viable.

The result of the HPLC-UV analysis of the naturally infected samples is shown in **Table 3**. Chaetoglobosins A and C,

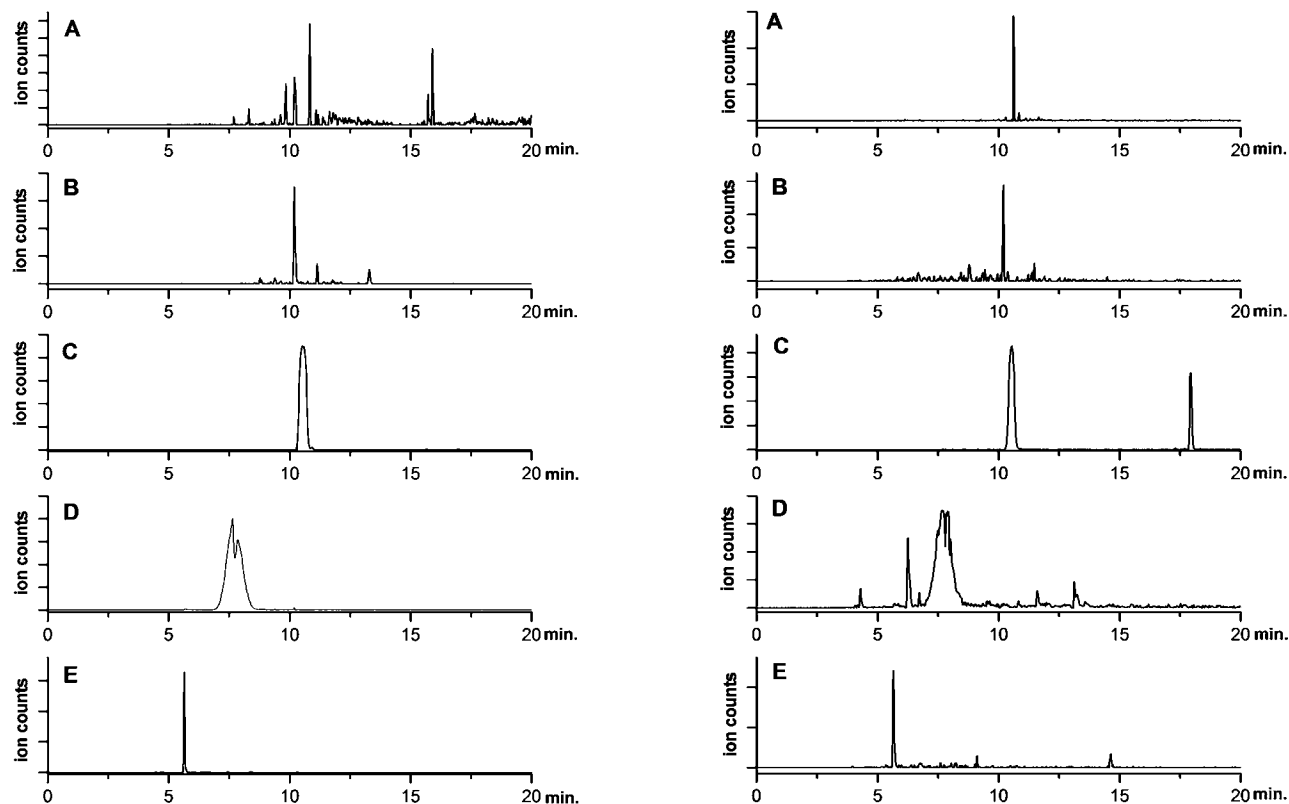
communisin B, and roquefortine C were detected in the moldy cherry juice, whereas only chaetoglobosin A and communisin B were detected in the moldy gooseberry juice. In the moldy potato pulp, which caused feed refusal in cows and thus had to be discarded by the farmer, chaetoglobosins A and C and communisin B were subsequently detected. In a moldy apple, infected with *P. expansum* while it was still on the tree, only chaetoglobosin A was detected. The moldy apple pulp was the only sample infected with fungi other than *P. expansum*. In this sample patulin was the only *P. expansum* metabolite detected; however, alternariol and alternariol monomethylether produced by *Alternaria* spp. were also detected. In the extract of windfall apples, picked from the ground during sampling, chaetoglobosins A and C, communisin B, patulin, and roquefortine C were detected. To illustrate the similarity of metabolite production on the windfall apples and *P. expansum* (BA 849) in pure culture, comparative samples were analyzed by LC-MS as shown in **Figure 2**. Narrow ion traces corresponding to the protonated masses of relevant metabolites (see above) were extracted from the chromatograms, and very similar chromatograms were obtained from the apple extract (right panel) and a pure culture extract (left panel).

## DISCUSSION

**Consistency in Metabolite Production.** The results from this study show that *P. expansum* is a consistent producer of chaetoglobosin A and communisin B and generally a consistent producer of patulin (98%) and roquefortine C (98%). Expansolides A and B were detected in 91% of the isolates that were tested. Citrinin was not as consistently produced, but was present in a majority of the 260 *P. expansum* isolates studied (72% of the TLC screened isolates compared to 85% of isolates analyzed using HPLC). It has been shown that gentisyl alcohol and 6-methylsalicylic acid are precursors for patulin (29) and suggested that aurantioclavine is a precursor for communisins A and B (30). This is substantiated by the fact that both aurantioclavine and gentisyl alcohol were found in 50% of the *P. expansum* isolates. It may be possible to detect more precursors if younger cultures are examined.

Another result from this study is that *P. expansum* produces a limited number of the many different metabolites that have been reported for this fungus. A report on penicillic acid production by *P. expansum* (14) could not be confirmed and neither could that on Raistrick phenol production (10). Only 4 of the 40 metabolites reported by Paterson et al. (7), Bridge et al. (5), and Paterson and Kemmelmeier (6), namely, citrinin, gentisyl alcohol, patulin, and roquefortine C, could be found in the *P. expansum* isolates examined in this study. None of the 260 isolates examined produced ochratoxins, penitrems, rubratoxin B, or any other known fungal metabolite in our collection (26), although several unknown metabolites were found in all extracts of *P. expansum*. It has not been possible to connect the three unknown metabolites found on TLC with the unknown ones detected by HPLC or suggest possible chemical identity, but because of their consistent production they should be structurally elucidated and tested for toxicity.

The results also show that the combination of metabolites produced by *P. expansum* is independent of geographic region and substratum and that this profile of metabolites is indicative for *P. expansum*. Although metabolites other than those detected in this study have been reported from *P. expansum*, these reports were often based on misidentified fungal isolates. For example, a *P. expansum* isolate (MRC 97 = CSIR 1029) was reported to produce griseofulvin and viridicatum-toxin (31, 32). This isolate



**Figure 2.** Narrow diagnostic ion traces from LC-MS analysis of *P. expansum* BA 849 in pure culture (left) and windfall apples naturally contaminated with *P. expansum* BA 849 (right): (A) chaetoglobosins E/F protonated mass at  $m/z$  531.286 width 30 ppm; (B) chaetoglobosins A–D and/or G protonated mass at  $m/z$  529.270 width 30 ppm; (C) communesin B protonated mass at  $m/z$  509.292 width 200 ppm; (D) communesin A protonated mass at  $m/z$  457.270 width 200 ppm; (E) roquefortine C protonated mass at  $m/z$  390.193 width 30 ppm. The apparent split peak in trace D of communesin A is due to significant saturation of the detector, resulting in a decrease of mass accuracy. The identities of the metabolites were in all cases confirmed by the ion pattern in the mass spectra as well as by retention times of standards.

was later identified as *P. aethiopicum* (10). Another *Penicillium* isolate, *P. aurantiogriseum* (CCF 2780), has also been reported to produce chaetoglobosin A (33). This isolate was re-examined and identified as *P. discolor*, which is another known producer of chaetoglobosins (15, 34). Chaetoglobosins, on the other hand, have never been found in any other isolate of *P. aurantiogriseum* or related species (35). Chaetoglobosin C has been reported from *P. aurantiovirens* (36), but the isolate producing this toxin was re-identified as *P. discolor* (34, 37). Apart from some as yet undescribed species of *Penicillium*, the communesins are produced only by *P. expansum* in the subgenus *Penicillium* (unpublished results).

**Detection Methods.** All methods used in this study are designed for qualitative detection of the metabolites, as coelution and lack of high-quality metabolite standards make quantitative multimetabolite analysis impractical, if not impossible. The different detection methods used show that not all metabolites can be detected equally well by the same method. TLC is well suited to rapid screening of citrinin, patulin, roquefortine C, and communesin B. These metabolites are usually produced in higher amounts compared to other *P. expansum* metabolites, and the reactions with spray reagents are sensitive. The TLC detection published by Larsen et al. (16) for expansolides A and B, where they appeared as an orange spot, could not be reproduced in this study. The use of HPLC-UV made it possible to detect metabolites produced in smaller amounts, such as chaetoglobosin A and communesin A. Citrinin, patulin, and communesin B are also easily detected due to their characteristic UV spectra. In the HPLC system used in this study, the alkaloids roquefortine C and communesin A elute as broad chromatographic

peaks, which interfere with the detection of other coeluting peaks/metabolites, for example, chaetoglobosin A. Hence, ethyl acetate was used as extraction solvent to reduce the amount of roquefortine C in the HPLC extracts. Methanol/chloroform (2:1, v/v) improves roquefortine C extraction if needed. Last, the terpenes expansolides A and B lack a chromophore with UV absorption above 205 nm and thus they are very difficult to detect by HPLC-UV.

**Natural Occurrence and Toxicity.** Chaetoglobosins A and C, communesin B, and roquefortine C were found in naturally contaminated fruit products, and an earlier study showed that cherry juice, artificially inoculated with *P. expansum*, contained patulin, chaetoglobosins A and B, communesins A and B, and expansolides A and B, but not citrinin (16). The chromatograms in Figure 2 clearly confirm the presence of roquefortine C, the communesins, and chaetoglobosins in the windfall apples and the *P. expansum* culture by comparing retention times with those of the standards, chromatographic peak shapes, and mass spectra including the accurate mass. Patulin, which was not detected in the MS systems used in this study, was detected in the windfall apple extract by HPLC-UV.

Individually, metabolites from *P. expansum* are known to have many different toxic effects. The chaetoglobosins have been reported to be orally toxic to 1-day-old cockerels (36) and to embryonic chickens (33), cytotoxic to HeLa cells, causing polynucleation (38), orally toxic to rats (39) and mice (38, 40), and teratogenic in mice, too (38). Citrinin has been reported to have nephrotoxic effects in mammals (41) and teratogenic effects in chickens (18). Communesin B is cytotoxic to P-388 lymphocytic leukemia cells (42). Patulin has shown to be

mutagenic, genotoxic, immunotoxic, and neurotoxic to rodents (4) and teratogenic to chickens (18), and roquefortine C is regarded as a neurotoxic in mice (43). The combined effects of *P. expansum* metabolites are, however, unknown. In this study, chaetoglobosins A and C, communesin B, and roquefortine C were found in one batch of industrially produced cherry juice, whereas one sample of windfall apples contained chaetoglobosins A and C, communesin B, roquefortine C, and patulin. Such samples, should they ever reach the consumers or end up as animal feeds, could have unforeseen toxicities, due to possible synergism between metabolites.

Chaetoglobosin A was a common denominator for all but one sample. Patulin, on the other hand, was only found in one sample. These results suggest that a patulin-negative sample is not always synonymous with a fungal metabolite-free sample. Chaetoglobosin A may thus be a better indicator of growth of the toxigenic *P. expansum* in fruit and vegetable products than patulin; however, chaetoglobosin A may itself also have health implications. Therefore, oral toxicological tests should be carried out on the chaetoglobosins together with other *P. expansum* metabolites and, if positive, methods should be developed that can detect at least patulin and chaetoglobosin A simultaneously, to increase the safety of fruit products.

## SAFETY

All work with fungal cultures was done in fume hoods to avoid exposure to fungal spores and volatiles. Preparation of metabolite standards and extractions of fungal cultures and moldy samples were done in fume cupboards and with the use of gloves.

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