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Production of Metabolites from the *Penicillium roqueforti* Complex

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Penicillium roqueforti comprises three accepted species: *P. carneum*, which is associated with meat, cheese, and bread; *P. paneum*, associated primarily with bread and silage; and *P. roqueforti*, which is associated with various processed foods and silage. This paper reports the use of HPLC-MS and HPLC-NMR to investigate the metabolites of silage-derived strains from two areas where silage toxicoses are regularly observed (Scandinavia and eastern Canada). Only modest differences were seen between the metabolites produced by strains from Canada and Scandinavia; however, silage strains of *P. paneum* isolated from Quebec were poor producers of patulin. This paper reports for the first time the production of festuclavine from *P. paneum*. This may be important as a possible explanation for the ill thrift observed when this species is dominant in poorly ensiled materials fed to dairy cows.

KEYWORDS: ***Penicillium roqueforti*; *Penicillium paneum*; *Penicillium carneum*; *Penicillium* metabolites; HPLC-MS; HPLC-NMR; silage; cattle toxicity**

INTRODUCTION

Penicillium roqueforti has long been reported in poorly ensiled crops as well as other habitats with limited oxygen, including wet wood. On the basis of morphology, molecular data, and secondary metabolite production this species has recently been separated into three taxa: *P. roqueforti*, *P. carneum*, and *P. paneum* (*1*). Starting with reports from Wisconsin in the early 1970s, consumption of such feed by cattle led to variable toxic outcomes. Occasionally the animals exhibited severe symptoms including hemorrhage and death; other cases were more associated with ill thrift. More recent studies in Canada and Scandinavia suggest that this effect is associated with the presence of *P. roqueforti* versus *P. paneum* (*1*, *2*). Despite many years of investigation, the toxic principles and the reason for the variation in response remain obscure.

P. carneum appears to be associated with meat products such as sausages, as well as cheese, bread, and barley (*3*). Isolates of this fungus consistently produce penitrems, patulin, and mycophenolic acid (**Figure 1**). *P. roqueforti* is the most common species of the three, and due to its resistance to organic acids and ability to grow at low pH, it is found as a contaminant of processed food such as bread, rye bread, beer, hard cheeses, and olives. Besides its use as a secondary starter culture in blue cheese, it is also commonly found in silage, grain, and other plant materials stored under microaerophilic conditions (*3*). It can produce a number of metabolites including mycophenolic acid and PR-toxin and its precursors, the eremofortines (**Table 1**) (*4*−*8*). These metabolites are not found in blue cheeses (*9*). As a newly described species, the natural habitats of *P. paneum* are not fully known. To date, bread and silage seem to be the most common sources of this species. Due to its much less pronounced sporulation compared with *P. roqueforti*, as well as mycologists formerly allocating *P. paneum* to *P. roqueforti*, the frequency of *P. paneum* is probably underestimated in studies on moldy silage. *P. paneum* strains always produce marcfortines, which is unique for it, typically also patulin, and under some conditions presumably also botryodiplodin (**Table 1**). Its morphological characteristics are also close to those of *Penicillium crustosum* when using the taxonomic treatment of *Penicillium* by Pitt (*10*), so it may have been confused with this species as well as with the two other members of *Penicillium* series *Roqueforti*.

There is limited information concerning the toxicities of metabolites associated with *Penicillium* series *Roqueforti*. Because of its use as a human drug (*11*), mycophenolic acid is best understood. It is antibacterial and immunosuppressive in animals and has been found to occur naturally in some blue cheeses and in silage (*12*−*14*). PR-toxin is lethal to rats, mice, and cats (*15*−*18*) and is mutagenic in the Ames test (*19*). Patulin is antibacterial and, in low doses, acts as a quorum-sensing inhibitor in *Pseudomonas aeruginosa* (*20*). In rats, patulin caused premature death after repeated oral dosing at ∼1.5 mg/

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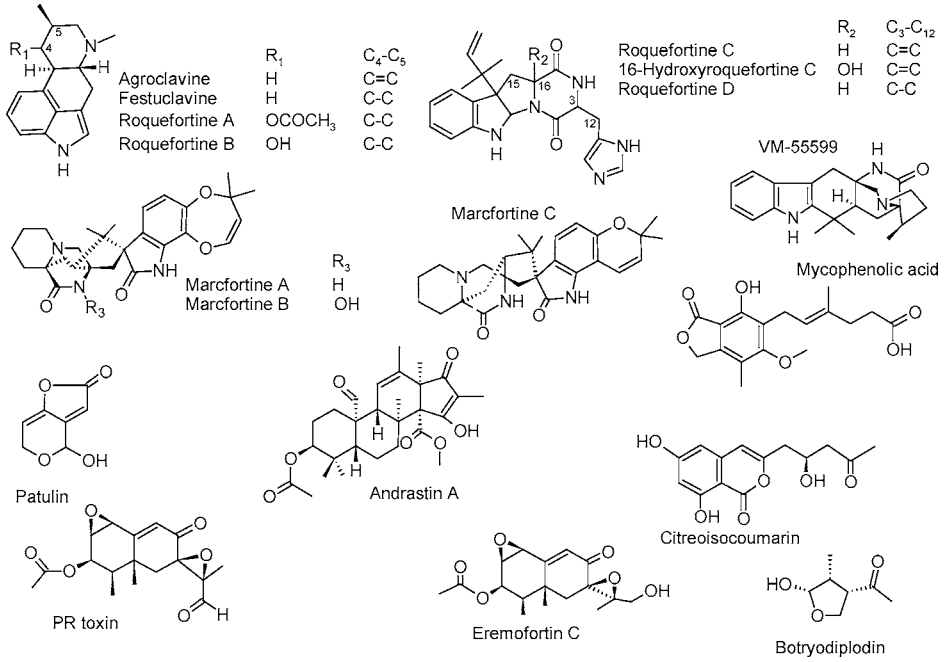

Figure 1. Structures of selected metabolites described from *P. roqueforti* and *P. paneum* in this and other studies.

Table 1. Mycotoxins and Other Secondary Metabolites Reported by Species in *Penicillium* Series *Roqueforti* Prior to This Study, Including Misidentified Species[a]

P. roqueforti	*P. paneum*	*P. carneum*
roquefortine C	roquefortine C	roquefortine C
roquefortine D	marcfortines A–C as *P. roqueforti* (*5*)	festuclavine as *P. crustosum* (*8*)
roquefortines A and B	patulin	roquefortines A and B as *P. crustosum* (*8*)
PR-toxin	orsellinic acid (*6*) as *P. roqueforti*	mycophenolic acid
eremofortins A–E	botryodiplodin as *P. roqueforti* (*7*)	patulin, as *P. roqueforti* and as *P. roqueforti* var. *carneum*
PR-imine (*4*)		penicillic acid, as *P. suavolens* and as *P. roqueforti*
PR-amide (*4*)		cyclopaldic acid
mycophenolic acid		penitrem A

[a] Adapted from Frisvad et al. (*3*, *31*), unless otherwise indicated.

kg of body weight (BW) (*21*) but otherwise is of low mammalian toxicity (*22*). Finally, marcfortines are moderately potent nematocides (*23*, *24*).

As noted, cases of intoxications of cattle have been ascribed to roquefortine C, and it is commonly found in poorly ensiled silage (*25*) as well as blue cheese (*9*, *26*). Despite this, the sparse data obtained so far suggest that it is not very toxic to animals. This and the fact that roquefortine is produced over a broad range of environmental conditions lead to the hypothesis that well-known acute toxicities associated with contamination by *P. roqueforti* were due to PR-toxin or other compounds (*2*). Roquefortine has antibacterial properties (*27*) and is Ames negative (*28*). There are no studies of its toxicity in laboratory animals that meet good laboratory practice (GLP) standards. Older data suggest roquefortine toxicity (ip) is in the range of 15−100 mg/kg of BW (*17*). Arnold et al. (*18*) report an oral LD_{50} of 169 mg/kg in male and 184 mg/kg in female CR57 mice and a similar value for male and female Swiss Webster mice. The data on neurotoxicity of roquefortine are equivocal. There are a number of incidents of dog toxicosis ascribed to roquefortine C (see, e.g., ref *29*), but penitrems or other compounds might have been present. At the doses tested (up to 50 mg/kg/day), no toxicity was seen in sheep, although the compound was absorbed and distributed widely through the tissues examined (*30*).

We decided to investigate the major and minor metabolites of *Penicillium* subgenus *Penicillium* section *Roqueforti* series *Roqueforti* (the *Penicillium roqueforti* complex) with an emphasis on silage-derived strains from two areas where silage toxicoses are regularly observed (Scandinavia and eastern Canada). This was achieved by growing many isolates on various laboratory substrates and analyzing the broth by liquid chromatography combined with on-line UV, high-resolution mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR).

MATERIALS AND METHODS

Chemicals and Media. Solvents used were of HPLC grade, and all other chemicals were of analytical grade unless stated otherwise. Yeast extract sucrose (YES), Czapek yeast extract (CYA), potato dextrose agar (PDA), creatine sucrose (CREA), oatmeal agar (OMA), and 0.5% malt extract agar (MEA) were produced as described elsewhere (*31*). Bacto 212750 yeast extract [Becton, Dickinson (BD), Sparks, MD] was used in YES and CYA and Bacto 218630 malt extract (BD) in MEA. Trace metals were added to all media (0.001% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$). Semisolid media additionally contained 2% agar. Water was purified from a Milli-Q system (Millipore, Bedford, MA). Reference standards of metabolites were available from previous studies in our laboratories, Sigma-Aldrich (Steinheim, Germany), Calbiochem, (San Diego, CA), and ICN (Irvine, CA) (*32*).

Fungal Isolates and Incubation. Strains tested are described in **Table 2**; details on the origin of the Canadian strains are found in ref *2*. For macromorphological identification (*31*, *33*) the strains were inoculated in 11 cm Petri dishes on YES, CYA, CREA, and MEA agars for 7 days at 25 °C in darkness. Macromorphological characters

Table 2. *Penicillium* Strains Investigated in This Study

species	IBT no.[a]	isolated from	country[b]	year[c]	no. in other collections[d]
P. roqueforti	5299	apple	RU		IMI 174718
P. roqueforti	6074	sugar beet silage	DK	<1993	
P. roqueforti[e]	6754	roquefort cheese	FR	1904	CBS 221.30; ATCC 10110; IMI 024313
P. roqueforti	14410	grass silage	SE	<1993	
P. roqueforti	14412	grass silage	SE	<1993	
P. roqueforti	16952	grass silage	NO	<1993	
P. roqueforti	16953	grass silage	NO	<1993	
P. roqueforti	24729	grass silage	CDN	2003	DAOM 232127
P. roqueforti	24748	grass silage	CDN	2003	DAOM 232126
P. paneum[e]	12407	rye bread	DK	<1993	
P. paneum	13929	baker's yeast	DK	1995	
P. paneum	16402	barley	CDN	<1993	NRRL 1168
P. paneum	19477	grain	SE	1990	CBS 167.91
P. paneum	20603	grass silage	SE	1997	
P. paneum	21613	grass silage	SE	1997	CBS 112295
P. paneum	24721	grass silage	CDN	2003	DAOM 232121
P. paneum	24722	grass silage	CDN	2003	DAOM 232123
P. paneum	24723	grass silage	CDN	2003	DAOM 232125
P. paneum	24728	grass silage	CDN	2003	DAOM 232120
P. carneum	6885	salami	DE	<1993	CBS 466.95
P. carneum[e]	6884	rye bread	DK	<1993	CBS112297, IMI 29304
P. carneum	6888	barley	DK	<1993	CBS112487
P. carneum	16948	grass silage	NO	<1993	
P. carneum	19478	sausage	DE	1978	CBS 390.78

[a] IBT culture collection at Center for Microbial Biotechnology, Lyngby Denmark. [b] CDN, Canada; DE, Germany; DK, Denmark; FR, France; NO, Norway; RU, Russia, SE, Sweden. [c] <1993 isolated before this data but year not known. [d] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM, Department of Agriculture, Ottawa, Mycology, Ottawa, ON, Canada; IMI, CABI Bioscience Genetic Resource Collection, Egham, U.K.; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, National Center for Agricultural Utilization Research, Peoria, IL; ATCC, American Type Culture Collection, Manassas, VA. [e] Type culture.

included colony diameter, color of the obverse and reverse, and pigmentation of medium on YES, CYA, and MEA, as well as acid production and thickness of the mycelium on CREA. YES, CYA, and PDA agar cultures were used for metabolite profiling and inoculated in the same way as the identification media.

A number of the strains of *P. paneum* and *P. roqueforti* recently isolated in Quebec (*2*) were also grown in 200 mL of liquid YES in 500 mL Erlenmeyer flasks incubated in still cultures at 25 °C for 14 days in darkness.

Extraction. *Liquid Cultures.* After incubation, cultures were filtered through a Whatman no. 1 filter (Brentford, U.K.). The filter cake was cut in pieces and shaken for 5 h two times with 200 mL of dichloromethane/methanol (9:1). The extract was subsequently filtered through a Whatman PS1 filter, evaporated in vacuo, redissolved in 5 mL of methanol, and filtered through a 0.45 μm PFTE syringe filter (Chromacol, Herts, U.K.).

The culture filtrate was extracted two times with 200 mL of dichloromethane, centrifuged at 4000*g* to break emulsions, and filtered through a Whatman PS1 filter. The combined extracts were subsequently evaporated in vacuo, redissolved in 5 mL of methanol, and filtered through a 0.45 μm PFTE syringe filter.

Agar cultures were extracted by cutting out 10 6-mm agar plugs that were transferred to a 4 mL vial and extracted two times with 2.5 mL of ethyl acetate (EtOAc), the first time overnight and the second time for ~3 h. The combined extracts were evaporated in vacuo, redissolved in 500 μL of acetonitrile (CH_3CN)/water (85:15 v/v), and filtered through a 0.45 μm PFTE syringe filter.

Liquid Chromatography−UV−High-Resolution Mass Spectrometry. LC-DAD-MS analysis using 1−5 μL of subsamples was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and a 50 × 2 mm i.d., 3 μm, Luna C_{18} II column (Phenomenex, Torrance, CA). The LC system was coupled to an LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.), with a Z-spray electrospray ionization (ESI) source and a LockSpray probe (*32*). The LC system was operated using water/CH_3CN gradient systems starting from 5−15% CH_3CN and increasing these to 100% CH_3CN in 16−20 min, maintaining this for 5 min (*34*).

Samples were analyzed in positive ESI (ESI^+) mode at a resolution of >6500 (at half-peak height) using two scan functions with different cone potentials as previously described (*34*). Water was buffered with 10 mM ammonium formate and 20 mM formic acid and CH_3CN with 20 mM formic acid. Samples were also analyzed in negative ESI (ESI^-) mode at a resolution of >5000 using two scan functions. Here the CH_3CN was unbuffered, and water was buffered with 100 μL/L formic acid (*34*). Leucine enkephalin was used as lock mass in both polarities.

Liquid Chromatography−NMR. LC-NMR was performed on a Varian 9010 HPLC system with UV detector attached to a Varian 600 MHz INOVA spectrometer using a 5 mm broadband cryoprobe with LC-NMR insert. Varian LCStar software controlled the coordination of the LC pump and the NMR probe. Experiments were performed in stopped-flow mode, allowing for ^1H spectrum acquisition of each LC peak before the gradient was continued using the previously described HPLC column and conditions (*2*).

Analysis of LC-DAD-MS and LC-NMR Data. UV, ESI^+, and ESI^- spectra from peaks not found in blank samples were matched against the reference standard database (~630 compounds) (*32*) as well as the library of spectra seen in other fungal extracts (~1000 spectra). Peaks still not identified were then matched against Antibase 2003 (~30000 microbial secondary metabolites) (Wiley & Sons, Hoboken, NJ). Compounds described in the literature from the species complex were coanalyzed, and the data files from the fungal extracts were then inspected by plotting the extracted ion chromatograms (± *m*/*z* 0.01) for the most predominant ion of the compounds of interest. Reference standards coanalyzed were cyclopiazonic acid, cyclopaldic acid, gentisyl alcohol, mycophenolic acid, orsellinic acid, patulin, penicillic acid, penitrem A, PR-toxin, roquefortines A−D, 16-hydroxyroquefortine C, benzoic acid, 3- and 4-hydroxybenzoic acids, *p*-anisaldehyde, pyrogallol, agroclavine I, festuclavine, secoclavine, citreoisocoumarin, 6-methylcitreoisocoumarin, marcfortines A and B, and andrastins A and E.

Metabolites not available as reference standards were tentatively identified by UV and HRMS data that were deconvoluted by plotting all ions found from background-subtracted MS spectra. Extracted ion chromatograms (± *m*/*z* 0.01) were also reconstructed for the following ions: $[M + H - H_2O]^+$, $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, and $[M + CH_3CN]^+$; $[M + CH_3CN+Na]^+$ (ESI^+ data files) as well as $[M - H]^-$ and $[M + HCOO^-]^-$ (ESI^- data files), calculated from components described from the three species in the literature. LC-NMR was used to provide confirmation for metabolites when no standards were available or in the cases when MS data were inconclusive. Additionally,

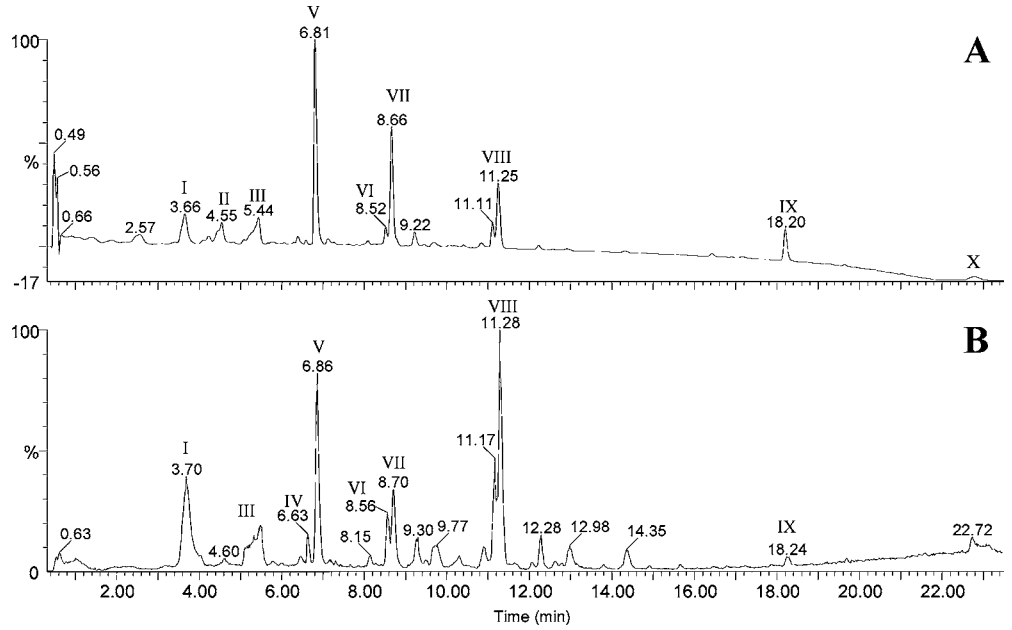

Figure 2. (**A**) HPLC (200–700 nm) chromatogram and (**B**) total ion positive electrospray chromatogram of *P. roqueforti* IBT14412 extract grown on yeast extract sucrose agar 25 °C for 7 days. Peaks of known metabolites: I, roquefortine A; II, citreoisocoumarin; III, partly coeluting eremofortin C, unidentified eremofortin, and roquefortine D; IV, 16-hydroxyroquefortine C; V, roquefortine C; VI, mycophenolic acid; VII, PR-toxin; VIII, andrastin A; IX, linolenic acid; X, ergosterol.

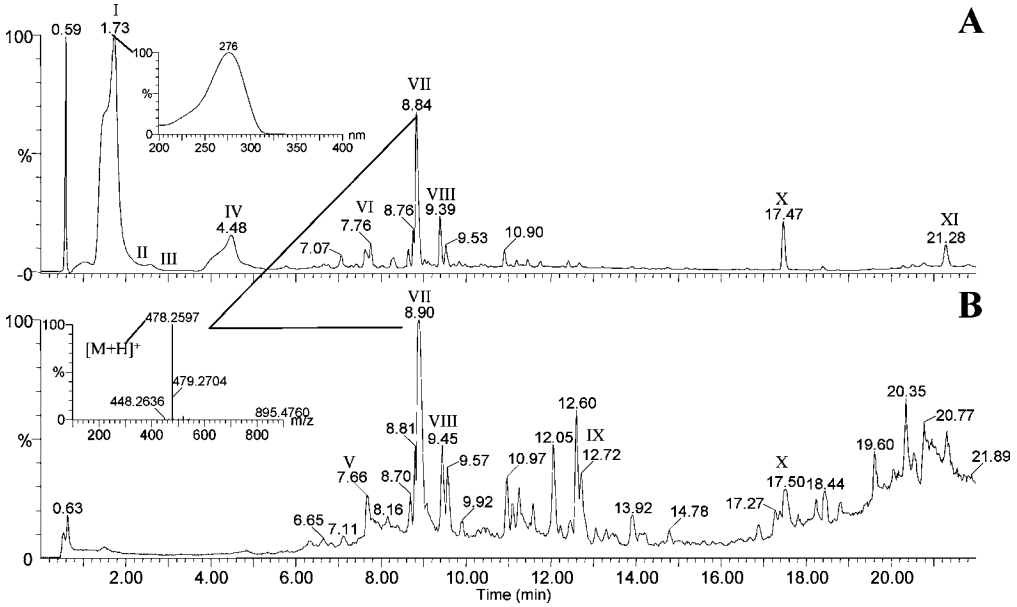

Figure 3. (**A**) HPLC (200–700 nm) chromatogram and (**B**) total ion positive electrospray chromatogram of *P. paneum* IBT12407 extract grown on yeast extract sucrose agar 25 °C for 7 days. Peaks: I, patulin; II, triacetic acid lactone; III, 2,5-dihydroxybenzoic acid; IV, 3-hydroxybenzoic acid; V, VM-55599; VI, citreoisocoumarin; VII, marcfortine A; VIII, roquefortine C; IX, andrastin A; X, linolenic acid; XI, ergosterol.

the larger volumes injected with LC-NMR allow for easy collection of individual peaks for further characterization and for use as MS standards.

RESULTS AND DISCUSSION

In our previous work on the *P. roqueforti* complex, Hypersil BDS-C_{18} and Nucleosil C_{18} columns were used (*1*, *32*). These are poorly deactivated compared to the Luna C_{18} II column used in this study, where 15−20 s peaks (**Figures 2** and **3**) could be obtained for the alkaloids compared, rather than the 2−3 min peaks obtained on the older columns when the interactions between the silanol groups and the N-groups were much stronger. This was probably why roquefortine C was not detected in *P. paneum* by Boysen (*1*). A limitation of the Luna C_{18} II phase is that mycophenolic acid and PR-toxin coelute in the acidic solvent systems used for both ESI^+ and ESI^- (50 μL/L trifluoroacetic acid in both solvents). Although the two compounds can easily be differentiated by UV, one or the other can be masked under the other when there are large differences in the relative amounts of the two compounds. They have the same composition, $C_{17}H_{20}O_6$; thus, high-resolution MS does not resolve this issue. Mycophenolic acid could be detected from its predominant $[M + NH_4]^+$ ion in scan trace 1 (low in-source fragmentation) or the *m*/*z* 207 in scan trace 2 (high in-source fragmentation). PR-toxin could be detected from scan trace 2, the fragment *m*/*z* 279, and the adduct *m*/*z* 428.16 [M + 44.025

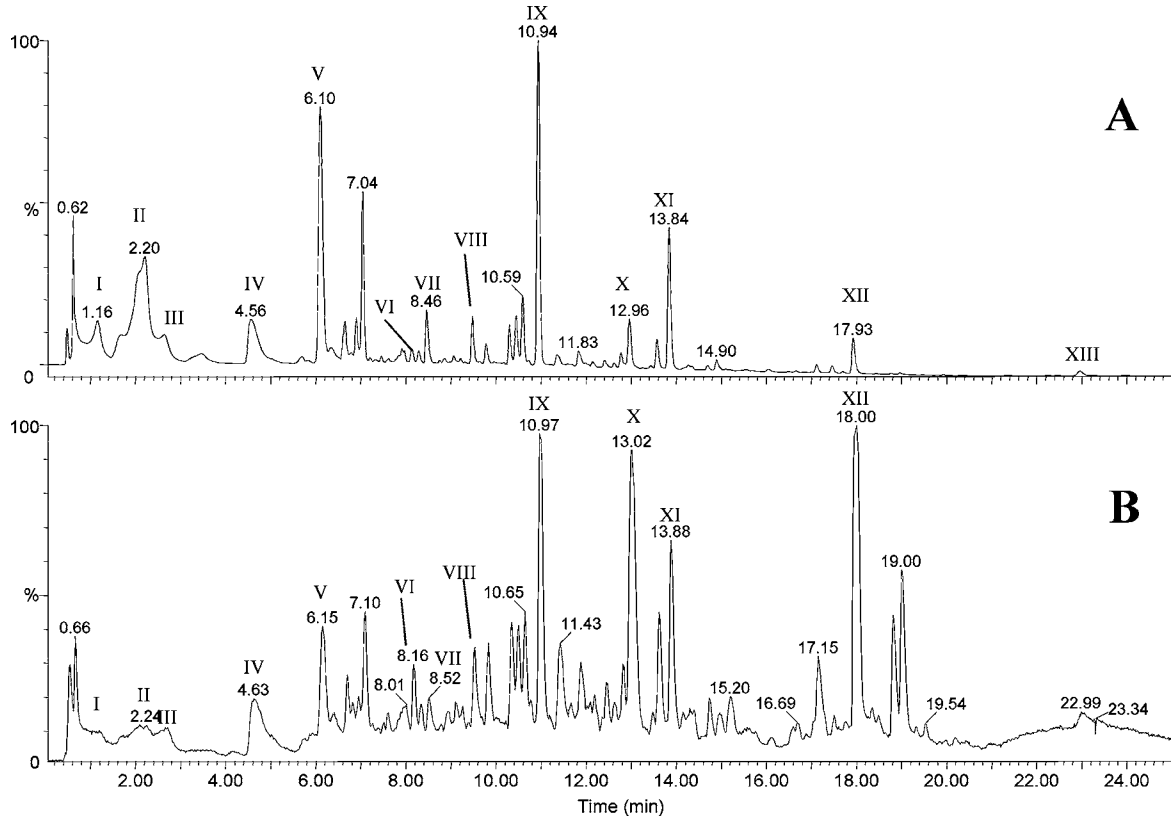

Figure 4. (**A**) HPLC (200–700 nm) chromatogram and (**B**) total ion negative electrospray chromatogram of *P. carneum* IBT 19478 extract grown on yeast extract sucrose agar 25 °C for 7 days. Peaks: I, gentisyl alcohol; II, patulin; III, triacetic acid lactone; IV, 2,5-dihydroxybenzoic acid; V, hydroxybenzoic acid; VI, citreoisocoumarin; VII, roquefortine C; VIII, 6-methylsalicylic acid; IX, mycophenolic acid; X, andrastin A; XI, mycophenolic acid analogue; XII, linolenic acid; XIII, ergosterol.

$+ Na + CH_3CN]^+$. If MS detection is not available, pH can be increased slightly to achieve separation between the two metabolites. The deconvoluted ^1H spectrum of each metabolite was also used to aid identification of the partly coeluting compounds.

As patulin and its precursors are very polar, it was necessary to increase the water content of the mobile phase to 95% (**Figure 3**) to obtain retention and subsequent separation from the void peak. However, then a maximum 1.5 μL of sample (in methanol or acetonitrile) could be injected without resulting in a prepeak of the most polar analytes as seen in **Figure 3**. Patulin and most of the benzoic acid derivatives could not be detected using ESI^+, and thus they were determined by ESI^- and their UV spectra (**Figure 4**). When only these metabolites are targeted, samples should be dissolved in the initial mobile phase and a more polar stationary phase such as phenyl or, even better, pentafluorophenyl (PFP), rather than the C_{18} phase column used. This provides a 5−10% higher content of organic in the mobile phase and thus better ionization.

No metabolites were produced on PDA cultures not seen on YES agar cultures (data not shown). Metabolites identified from agar cultures are shown in **Table 3**. Broadly, all three species produced roquefortine C as well as roquefortines A and D in lesser amounts. In the strains tested, the related metabolite 16-hydroxyroquefortine (*35*) was detected in relatively larger amounts in *P. roqueforti* and in lower quantities in *P. paneum*. Other metabolites common to all three species included andrastins A and B, orsellinic acid, and citreoisocoumarin. Much more of the latter two (5−10×) was produced when the cultures were grown on manganese-enriched PDA (data not shown). This is known to enhance patulin production in *P. expansum*, *P. griseofulvum*, *P. clavigerum*, and *P. coprobium* (*36*).

Patulin was the sole metabolite produced by *P. paneum* and *P. carneum* but was not produced by *P. roqueforti*. Of the Canadian strains tested, one strain isolated from barley in Ottawa (IBT 16402) was a good producer of patulin, whereas only one (IBT 24722) of the four strains isolated from various ensiled material in Quebec produced this compound, even when grown on manganese-enriched PDA (data not shown). However, some of strains did produce moderate amounts of the precursors 2,5-dihydroxybenzoic acid, triacetic acid lactone, genticyl alcohol, and 3-hydroxybenzoic acid (data not shown). We were not able to induce production of botryodiplodin in detectable levels from the *P. paneum* cultures even on the manganese-enriched PDA, where its precursor orsellinic acid was enhanced. Successful production by *Botryosphaeria rhodina* (CBS 356.59) was used as a positive control.

P. roqueforti and *P. carneum* had a number of metabolites in common, including agroclavine, festuclavine, and mycophenolic acid. Among these species, penitrem A was produced solely by *P carneum*, marcfortines (A, B, C) and their precursor VM-55599 were accumulated only by *P. paneum*, and PR-toxin and its precursor emerfortine C were produced solely by *P. roqueforti*.

The strains isolated from Quebec silage tested in liquid YES produced metabolites similar to those from the corresponding agar plates. However, there were some differences (**Table 4**). More of the minor roquefortine derivatives were seen in either the culture filtrate or the mycelium. In addition, several Canadian strains of *P. paneum* produced festuclavine in liquid cultures, something not found in the agar cultures and not previously reported for this species.

Consistent with previous studies, and regardless of geographic origin, *P. roqueforti* strains produce mycophenolic acid and/or

Table 3. Metabolites Detected from *P. carneum* and *P. roqueforti* Strains Grown on Yeast Exact Agar[a]

species IBT no.	country	agro-clavine	festud-avine	roque-fortine C	roque-fortine A[a]	roque-fortine D	16-OH-roque-fortine	patulin	orsellinic acid	peni-trem A	VM-55599	marc-fortine A	marc-fortine B	marc-fortine C	andrastin A	andrastin B	citreo isocoum-arin	PR-toxin	eremo-fortin C	myco-phenolic acid
P. carneum 6884	DK		++	++	++	+		++		++					++		++			+++
P. carneum 6885	DE		+	++	++	+		+++		++					++	+	++			+++
P. carneum 6888	DK		++	++	++	++		+++		++					++	+	+++			+++
P. carneum 16948	NO		+	++	+	++		++	+++	++					++	+	+++			+++
P. carneum 19478	DE	+	++	++	++	+		++	+	+					++	+	++			+++
P. paneum 12407	DK			++		+	+	+++			++	++	++	+	++	+	++			
P. paneum 13929	DK			++				++	+		++	++	++	+	++	++	++			
P. paneum 19477	SE			+				++	+++	++	++	++	++	++	++		++			
P. paneum 20603	SE			++			+				++	+++	++	++	++	+	++			
P. paneum 21613	SE			++				+++	+		++	+++	++	++	+++	++	++			
P. paneum 16402	CDN			++				+++			++	+++	++	++	++	+	++			
P. paneum 24721	CDN			+					++		++	+++	++	+	+++	++	+++			
P. paneum 24722	CDN			+					++		++	++	++	+	++	+	+++			
P. paneum 24723	CDN			++						++	++	+++	++	++	++	++	+++			
P. paneum 24728	CDN			+					+++		++	++	++	+	++	++	++			
P. roqueforti 5299	RU	+	++	++	++	+	+		++						+++	++	+++	++	++	+++
P. roqueforti 6074	DK		++	+++	+++	++	++		+						+++	++	++	++	++	+++
P. roqueforti 6754	FR	+	++	+++	++	+	+								++		++	++	++	++
P. roqueforti 14410	SE	++	++	++	++	++	++		+						+++	++	++	++	++	
P. roqueforti 14412	SE	++	++	++	+	++									+++	++	++	+++	++	++
P. roqueforti 16952	NO	++	++	+++	++	++	+								+++	++	++	+	+	++
P. roqueforti 16953	NO	+	++	+++	++	++	+								+++	++	++	+++	++	+++
P. roqueforti 24729	CDN	+	++	+++	++	+									+++	++	++	++	+	++
P. roqueforti 24748	CDN	++	++	+++	++										+++	++	++		+++	++

[a] See **Table 2** for country identifications. +++, 10–100 μg/mL medium; ++, 1–10 μg/mL; +, = 0.0–1 μg/mL. Roquefortine A is also known as isofumigaclavine A.

Table 4. Selected Metabolites Detected from Canadian *P. roqueforti* and *P. paneum* Strains Grown in Yeast Extract Sucrose Media[a]

species IBT no.	festuclavine	roquefortine C	roquefortine A	roquefortine B	roquefortine D
mycelium, still					
P. paneum 24721		+++			+
P. paneum 24723		+++			+
P. paneum 24728	+	+++	+		+
P. roqueforti 24749	+++	+++	+++	++	++
P. roqueforti 24748	++	+++	+++		++
culture filtrate, still					
P. paneum 24721		+++		+	
P. paneum 24723		+++			
P. paneum 24728		+++	+	+	
P. roqueforti 24729	+++	+++	+++		++
P. roqueforti 24748	++	+++	+++		++

[a] +++, 10–100 mg/mL medium; ++, 1–10 mg/mL; +, 0.0–1 mg/mL. Roquefortines A and B are also known as isofumigaclavines A and B, respectively.

PR-toxin. *P. paneum* appears to be more variable in its ability to produce patulin and intermediates such as isoepoxydon, despite the fact that the first precursors (6-methylsalicylic, 2,5-dihydroxybenzoic, and 3-hydroxybenzoic acids) were produced by many strains (data not shown). As previously reported (*31*) marcfortines were consistently and uniquely produced by *P. paneum* (confirmed by MS and LC-NMR). The metabolite VM-55599 was also found in *P. paneum*. This was isolated from *Penicillium* sp. IMI 332995 (*P. brasilianum* Batista; Frisvad, unpublished data) and some other fungi. VM-55599 is thought to be a biosynthetic precursor of brevianamides and marcfortines (*23*, *37*). In the previous paper by Boysen et al. (*1*), marcfortines were misinterpreted as eremofortines in cultures of *P. roqueforti*.

This study has expanded our knowledge of the metabolites from this species, particularly with respect to strains from agricultural areas in eastern North America. The three species in *Penicillium* series *Roqueforti* are physiologically and morphologically closely related, yet they can be distinguished by particular nucleotide sequences (*1*, *38*, *39*), macromorphological features, and their production of secondary metabolites (**Table 3**). Sumarah et al. (*2*) suggested that roquefortine had become regarded as the toxic metabolite because it is common within the group and produced over a broad range of environmental conditions. They noted that the best documented cases of illness associated with severe toxicoses were with PR-toxin. This metabolite may also occur in feed in the parts of the United States where dairy cows are fed silage (*40*). Both the Canadian and Scandinavian studies indicate that *P. roqueforti*, which uniquely produces PR-toxin in the series, was associated with more severe toxicosis. *P. paneum*, which produced the alkaloid festuclavine (along with *P. roqueforti*), was associated with ill thrift. This may be important as these and similar compounds are associated with cattle and horse ill thrift resulting from consumption of endophyte-contaminated grasses (*41*, *42*). Moreover, festuclavine has been detected in 8 of 10 moldy Danish silage samples, where the concentration appeared to correlate with marcfortine A concentration (Nielsen, unpublished data).

It remains possible that interactions between the various toxins produce the toxicities observed, for which there is a model in the toxins from *Fusarium graminearum*. Grain contaminated by this fungus is usually more toxic than the dexoynivalenol concentration would indicate; several metabolites co-occur with deoxynivalenol in approximately similar concentrations, and there are indications that these interact (*43*, *44*). However, as far as is known, this does not occur unless each compound is above its effect threshold. Not enough is known about the actual occurrence of the metabolites discussed in the present study in silage to comment on this possibility further.

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