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Toxins from strains of *Penicillium chrysogenum* isolated from buildings and other sources

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Abstract

In 2004, Scott et al. (*Mycologia* 2004; 96: 1095–1105) determined that there are four molecular species within *P. chrysogenum*, one of which (clade 4) was dominant in isolates in house dust in ~100 homes in southern Ontario, Canada. We collected additional strains from buildings across Canada and obtained some from DAOM. The large majority of our strains were in clade 4, with a modest number of strains in Clade 1. Because these strains came from across Canada, the dominance of clade 4 in buildings is apparently widespread. Most strains tested produced penicillin G, roquefortine C and unexpectedly, meleagrin in high yield. Additionally, there appeared to be strains differentiated by their ability to accumulate xanthocillin X. These studies allowed focused toxicity studies in vivo and with primary lung cell cultures to be undertaken on the basis of reliable information of the toxins that should be studied.

Key words: built environment, clade 4, metabolites, *Penicillium chrysogenum*

Introduction

Penicillium chrysogenum is one of the most common fungi growing on damp building materials in North America and Western Europe [1–3]. As a result, it is also common in house dust and comprises an important bioaerosol exposure in damp homes. Damp and moldy housing is linked with higher incidences of asthma, and, with increased upper respiratory disease [4]. The former response is allergic in nature, whereas the latter is a toxic response.

Profiles of secondary metabolites and DNA sequencing of ribosomal and protein coding genes have been used to clarify phylogenetic relationships of moulds as well as for classifying species [5]. Advances in metabolite analysis have allowed the use of secondary metabolite profiling for fungal identification. Frisvad and colleagues have made extensive use of this technique to facilitate identification of *Penicillium* species that are difficult to recognize by morphology alone. Examples include the separation of *P. carneum* and *P. paneum* from *P. roqueforti* [6]. In contrast, *Stachybotrys chartarum* was split into *S. chlorohalonata* and *S. chartarum* on the basis of molecular and morphological differences despite overlapping patterns of secondary metabolites [7].

In a recent monograph of the subgenus *Penicillium*, Frisvad and Samson [8] placed *P. chrysogenum* in the section Chrysogena series Chrysogena, all the species of which produce penicillin. *P. chrysogenum* and *P. flavigenum* have similar metabolite patterns, but the remaining species have apparently distinct principal metabolite patterns. Other species included were *P. flavigenum*, *P. diposomyis* and *P. nalgiovense* II. In the same year, Scott et al. [9] studied ~200

isolates of *P. chrysogenum* recovered from dust samples in 109 houses in southern Ontario, as well as culture collection isolates and ex-type strains. Genotypes were determined by analysis of partial DNA sequences of acetyl co-enzyme A synthase, beta-tubulin, thioredoxin reductase and the internal transcribed spacer regions of the nuclear ribosomal subrepeat. Three strongly supported lineages were resolved; one containing more than 90% of the indoor isolates that also included the Fleming penicillin strain. A second clade containing more than 5% of house isolates clustered with the ex-type strains of *P. chrysogenum*. One consequence of these findings is that the morphospecies *P. chrysogenum* is, by molecular measures at least, four species, only one of which may occur in building materials. The example noted above of *P. roqueforti* is a case where what was *P. roqueforti* sensu lato comprised strains that produced different toxins [10].

As part of a study of the allergens and toxins of damp building-material associated fungi, we investigated strains of *P. chrysogenum* isolated from moldy building materials to determine the variation in the toxigenic potential of these strains. This was done for the purpose of assessing whether the metabolites from the building-associated clade were distinct. The intent was to reliably determine which toxins should be tested in toxicity studies based on modern studies of the extrolites in section Chrysogena series Chrysogena [8]. Culture conditions were chosen to favor the accumulation of the known metabolites of this taxon including penicillin.

Materials and methods

Cultures

Cultures of *P. chrysogenum* were obtained from DAOM (Agriculture & Agri-Food Canada, Ottawa) and the identities of all were confirmed by morphological [8] and molecular analyses [9]. Additional strains were obtained from indoor air or moldy building materials provided by Paracel Laboratories Ltd. (Ottawa, Ontario). The cultures from DAOM were transferred aseptically to sterilized 2% malt extract (Difco) agar slants (MEA; 20 ml tubes, 5 ml of agar, cooled on a slant producing a standard surface area of 424 mm^2). These were incubated at 25 °C for 7 days and stored at 5 °C for >2 months. Building material isolates were transferred from the laboratory's MEA identification plate (i.e. after one transfer) to new MEA plates. Single spore isolates were prepared and transferred to MEA slants and then deposited in DAOM. The origin of the strains is listed in Table 1.

Molecular characterization

Partial sequences of the β-tubulin gene were generated for *P. chrysogenum* strains DAOM 234051-234067 using DNA isolated from conidia and mycelia produced on Blakeslee's malt extract agar using the UltraClean Microbial DNA Isolation (Mo Bio Laboratories Inc., Solana Beach, CA, USA) kit. Polymerase chain reactions (PCR) were performed in 25 μl volumes using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway NJ, USA) and 2 μl of template, using a Techne Genius thermocycler (Techne Inc., Burlington, NJ, USA). PCR cycling parameters included 30 cycles of denaturation at 95 °C for 1.5 min, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min, with an initial denaturation of 4 min and a final extension step of 10 min. Some amplified products were purified using the UltraClean Microbial PCR Purification Kit (Mo Bio Laboratories) and DNA concentrations were estimated from fragments stained by ethidium bromide and separated by agarose gel electrophoresis. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA, USA) with the recommended cycling parameters. Reactions were purified by ethanol/sodium acetate precipitation. The sequences were determined using an ABI PRISM™ 3100 DNA automated sequencer (Applied Biosystems, Foster, CA). Exons 3–6 of the β-tubulin gene were amplified and sequenced using primers Bt2a and Bt2b [11]. Consensus sequences were determined from overlapping sequence data for both DNA strands, except where noted, using the software Sequencher (Gene Codes Corp., Ann Arbor, MI, USA).

Strains were typed by comparison with the β-tubulin sequences for *P. chrysogenum* in Scott et al. [9] using a pair-wise alignment comparison in

Table 1. Penicillium chrysogenum strains tested for metabolite production

DAOM	Year	Source
59494C	1990	*Substr. et loc. Incert*
155627	1976	Paper, Canadian Conservation Institute, Ottawa
155628	1976	As above
167036	1977	Spruce forest soil, Lacolle, QC
171025	1979	Salami, AAFC, Ottawa
175157	1980	Urea formaldehyde foam insulation, Niagara Falls, ON
175758	1980	Indoor air, Montreal
178623	1980	Paper product, QC
190864	1984	Stored barley, Winnipeg
193710	1990	Cheese, CT
212031	1990	Wood studs, Edmonton
215336	1990	As above
215337	1990	Hemlock lumber, Vancouver
234051	2003	Indoor air, Saskatoon
234052	2003	Pipe wrap, Ottawa
234053	2003	Indoor air, Ottawa
234054	2003	House dust, Calgary
234055	2003	Outdoor air, Ottawa
234056	2003	Gypsum wallboard, Calgary
234057	2003	House dust, Deseronto, ON
234058	2003	Indoor air, Saskatoon
234059	2003	Insulation, Surrey, BC
234060	2003	Outdoor air, Ottawa
234061	2003	Indoor air, Carp, ON
234062	2004	Gypsum wallboard, Ottawa
234063	2004	Wood, Ottawa
234064	2003	Gypsum wallboard, Ottawa
234065	2003	Unknown building material, Ottawa
234066	2003	Air sample, Sudbury, ON
234067	2003	Indoor air, Ottawa

the *Penicillium* subgenus *Penicillium* section of MycoBank (www.mycobank.org).

Fermentations

Slants of individual strains were macerated in 50 ml of sterile distilled water, and an aliquot (5% v/v) used to inoculate three Roux bottles each containing 200 ml of one of two media. To assess penicillin production, Czapek-Dox medium supplemented with 1% corn steep solids (Fermtech) and phenoxyacetic acid (Sigma) was used [12]; for the remaining metabolites, Czapek-Dox supplemented with 5% yeast extract (Sigma) [13] was prepared. The Roux bottles were covered in aluminum foil and incubated in the dark at 25 °C for 14 days. Cultures were filtered through a Buchner funnel using Whatman #1 filter paper under vacuum, the recovered volume and pH were measured and the filtrates were stored at 5 °C. The resulting mycelium was rinsed twice with equal volumes of distilled water, dried under vacuum, freeze-dried and ground to a fine powder.

Analysis

The analysis of the penicillin fermentations followed the methods of Anne [12]. Briefly, the pH of the culture filtrates was adjusted to 2.5 with 1 N HCl. The filtrates were then extracted with 200 ml of butyl acetate (J.T. Baker, USA) in separatory funnels. The butyl acetate phase was extracted in 5 ml of phosphate buffer (30 mM, pH 6.0) and 15 ml of 5% (w/v) $NaHCO_3$, resulting in a final pH of 6.0. To remove any water, the butyl acetate extracts were dried by the addition of approximately 10 g of Na_2SO_4. The extracts were then concentrated under vacuum, re-suspended in butyl acetate and dried under nitrogen gas in tared vials. The mycelium was extracted with 33 ml butyl acetate/g wet weight mycelium in 1 l Erlenmeyer flasks and stirred for 8 h. They were dewatered by

filtering through Na_2SO_4, and dried to completion under vacuum. Each sample was re-suspended in butyl acetate and dried under nitrogen gas in vials.

Penicillin concentrations were determined using an Agilent 1100 series HPLC with a quaternary pump with a diode array detector connected to a Synergi Max-RP, 250×4.6 mm^2, 4 μm, 80 Å HPLC column (Phenomenex, California). A gradient mobile phase was used consisting of a 30 mM K_2HPO_4/KH_2PO_4 (50:50) mixture (A) and acetonitrile (B) at a flow rate of 1 ml/min and pH 5.5. The HPLC program ran at 100% (A) for 16 min changing to 80% (A) and 20% (B) in 15 min, holding at this percent for 5 min and then returning to the initial conditions of 100% (A) for a further 5 min. The detector was monitored at 220 nm, which was found to be optimal for penicillin. All extracts were filtered through 13 mm PTFE (0.45 μm) syringe filters prior to injection. A penicillin G standard was obtained from Sigma (St. Louis, USA).

For the analysis of the other metabolites, the culture filtrates at pH 6, were extracted twice with equal volumes of ethyl acetate (Fisher Scientific, Ottawa) in separatory funnels. The combined extracts were dried using Na_2SO_4 and concentrated under vacuum and stored in vials. The mycelium was extracted in ethyl acetate and the final extract dried under nitrogen as above. Because roquefortine C is light sensitive, these cultures were filtered and extracted in darkness and all samples stored in amber vials. Determination of the other metabolites followed the methods of Nielsen & Smedsgaard [14]. Using the equipment and column described above, a gradient mobile phase consisting of an acetonitrile/0.05% TFA mixture (A) and water (B) at a flow rate of 1 ml/min was used. The HPLC program ran at 15% (A), 85% (B) for 40 min, going to 100% (B) in 5 min changing to 85% (A), 15% (B) in 8 min and held at 85% (A), 15% (B) for an extra 5 min before returning to the initial conditions. The detector was monitored at 215, 254 and 354 nm. Standards of meleagrin and roquefortine C were prepared in our laboratory [15] and small amounts of sorbicillin and xanthocillin X were a gift from K.F. Nielsen. The latter turned out to be a major metabolite and a sufficient amount was purified to ensure that our identifications by mass spectroscopy were correct and to enable the acquisition of quantitative data.

Recovery studies

Aqueous stock solutions (5 mg/ml) of penicillin G, meleagrin, roquefortine C and xanthocillin X were serially diluted to create standard calibration curves. A range of concentrations from 1 to 4 mg/ml, representing 10–50 μg of penicillin G on the column was used.

For recovery studies, 50 ml of the penicillin test medium (Czapek-Dox medium supplemented with 1% corn steep solids) was spiked with 5, 10 and 30 μg penicillin and analyzed as above. The remaining metabolites were spiked into the Czapek-Dox supplemented with 5% yeast extract medium at 1, 10 and 20 μg. All of the recovery experiments were performed in triplicate. The available xanthocillin X and sorbicillin standards were insufficient for a recovery study.

LC-MS, LC-MS/MS and NMR analysis

LC-MS was used to identity metabolites from both the filtrate and mycelium. The instrument was an HP 1050 HPLC system attached to a Quattro LC-triple quadropole electrospray ionization (EI) mass spectrometer (Micromass, UK). The instrument was set for analysis in positive ion mode, with full scan spectra between m/z 200 and 1000. The EI-MS was controlled using MassLynx software version 3.5, which was also used for analysis of mass spectrum data. This allowed for the analysis of extracts under identical HPLC conditions to those described above. The effluent was divided using a micro-splitter valve (Upchurch Scientific, WA), such that the flow to the electrospray was approximately 100 μl/min. This was diverted through capillary tubing set at −3.8 kV into the EI-MS. The source block temperature was 80 °C and the cone voltage was −47 V. Nitrogen served as the drying and nebulizing gas at a flow rate of 70 l/h, 5.5×105 Pa. The majority of the flow was diverted into the UV detector, as usual, to provide a chromatogram of the sample mixture.

NMR spectra were acquired on a Bruker ARX500 spectrometer at 500.13 MHz (1H) or 125.18 MHz (13C) operating at 303 K, using a 5 mm inverse triple (H/C/N) probe. NMR spectra were determined in acetonitrile-d3 (CD3CN) and chemical shifts were referenced to the solvent at 1.94 ppm for 1H and 1.39 ppm for 13C and are reported relative to tetramethysilane (TMS).

Table 2. Strains of *P. chrysogenum* that produced one or more metabolites (ug/g/l)

DAOM	Meleagrin	Roquefortine	Penicillin G	Xanthocillin X	Scott clade
59494C	–[a]	–	–	–	1
155627	4.21 ± 0.39	1.33 ± 0.05	–	–	4[b]
155628	4.75 ± 0.42	1.6 ± 0.81	2020.01 ± 148.2	–	4[b]
167036	5.52 ± 0.44	14.62 ± 0.99	–	–	4[b]
171025	7.1 ± 0.61	938.32 ± 68.3	7020 ± 523	–	1[b]
175157	54 ± 4	–	–	–	4[b]
175758	11.43 ± 0.92	–	3360.12 ± 225	–	4[b]
178623	3.9 ± 0.22	1.41 ± 0.08	1980.1 ± 112	–	1[b]
190864	12.7 ± 0.70	17 ± 1.1	–	–	4[b]
193710	–	–	–	–	1
212031	2.9 ± 0.1	6.2036	–	–	4[b]
215336	53.74 ± 2.9	145.58 ± 8.1	4786.26 ± 245.3	-	4[b]
215337	–	593.82 ± 41.3	4644.31 ± 233.3	–	4[b]
234051	23.03 ± 1.10	–	4656.11 ± 211.2	7.62 ± 0.51	**1**
234052	298.6 ± 22.6	–	6178.14 ± 489.3	426.86 ± 25.31	**4**
234053	–	254.64 ± 13.5	–	180.09 ± 10.24	**1**
234054	–	297.99 ± 12.8	–	232.73 ± 9.97	**4**
234055	136.69 ± 10.1	550.67 ± 39.2	5137.53 ± 332.9	106.5 ± 6.1	**1**
234056	15.65 ± 0.62	767.06 ± 68.1	5789.32 ± 358.9	120.48 ± 5.24	**4**
234057	107.41 ± 6.97	-	7028.06 ± 522.3	123.42 ± 7.31	**4**
234058	–	–	9672.23 ± 548.3	–	**4**
234059	–	5.01 ± 0.36	12732.72 ± 998	115.2 ± 8.2	**4**
234060	–	–	25361.34 ± 1025	–	**4**
234061	0.11 ± 0.05	152.34 ± 7.88	–	–	**4**
234062	–	87.58 ± 5.44	17652.24 ± 1048	–	**4**
234063	–	65.41 ± 2.1	–	–	**4**
234064	–	14.49 ± 1.00	–	–	**1**
234065	57.55 ± 4.88	51.51 ± 3.90	–	–	**4**
234066	–	251.16 ± 15.6	–	77.09 ± 4.21	**4**
234067	139.03 ± 7.3	41.98 ± 2.81	–	147.18 ± 8.3	**4**

[a] Not detected.
[b] Scott et al. [9]; values in bold from present study.

Chemical shift assignments were made with 1H/1H (COSY), and 1H/13C (HMQC, HMBC) inverse correlation spectra using standard Bruker pulse sequences. The remaining instruments used are described elsewhere [15].

Results

Fourteen of the strains were typed in group 4, and three in group 1, as defined by Scott et al. [9]. The double-stranded portions of the newly generated β-tubulin sequences were 426 bp long. The sequence matches were 100% in all cases, compared to reference sequences for these groups (group 1: AY371552, AY371553, AY371576; group 4: AY371550, AY371600).

The standard calibration curve of penicillin G was prepared by plotting the peak area counts versus the amount of penicillin G on the HPLC column (μg). The standard curve developed from the final gradient program showed a linear relationship with an R^2 value of 0.99 over the full concentration range used. These steps were repeated for roquefortine C ($R^2 = 0.98$) and meleagrin ($R^2 = 0.99$). Using the equations from the respective standard calibration curves, the percent recovery of penicillin G, roquefortine C and meleagrin from the spiked media extracts was determined to be 84.11 ± 4.17%, 84.67 ± 3.72% and 76.33 ± 2.59%.

All strains grew well on both media and there were no significant differences in mycelial mass among the strains (data not shown). The cultures produced varying amounts of meleagrin, roquefortine C, penicillin G and xanthocillin X (Table 2); all metabolites were confirmed by LC-MS and 1H NMR. Despite growing well,

metabolite yield was nil to low in seven strains that had been in the culture collection for some time (of 11). This was also the case for two freshly isolated strains (of 17). In these tests, one strain (DAOM 234060) isolated from gypsum wallboard produced only penicillin G. The remainder separated into two groups that were primarily differentiated by the accumulation of penicillin G. There was also evidence for strains that accumulate more versus less meleagrin (i.e. the distribution of the meleagrin positive values was not normal; Table 2).

Meleagrin was isolated as a light yellow solid, as follows: melting point, 251 °C; $[\alpha]_D$-225° ($CHCl_3$). High Resolution Mass Spectrum found 433.1729 (calculated for $C_{23}H_{23}N_5O_4$, 433.1752). IRυ_{max} ($CHCl_3$, cm^{-1}): 3161 (OH), 3009 (CO_2R), 1696 (CONH), 1668, 1648, 1614, 1439, 1394, 1353, 1310, 1218, 1108, 979, 751. MS (EI) *m*/*z* (rel. int.): 433 ($[M]^+$, 8), 365 (38), 364 (50), 318 (17), 277 (9), 86 (70), 84 (100), 47 (19), 41 (11), 31 (9), 29 (8).

Roquefortine C was isolated as a very fine feathery solid, as follows: melting point, 195–200 °C; $[\alpha]_D$-700° ($CHCl_3$). High Resolution Mass Spectrum, found 389.1861 (calculated for $C_{22}H_{23}N_5O_2$, 389.1854). IRυ_{max} ($CHCl_3$, cm^{-1}): 3197 (NH), 2971, 1681 (CONR), 1606, 1483, 1433, 1357, 1316, 1239, 1215, 1102, 921, 751, 643. MS (EI) *m*/*z* (rel. int.): 389 ($[M]^+$, 27), 321 (20), 320 (100), 319 (4), 198 (8), 192 (7), 161 (22), 158 (6), 150 (8), 143 (17), 131 (5), 130 (50), 112 (7), 108 (11), 107 (5), 70 (9), 69 (9), 50 (5), 41 (15), 31 (14).

Xanthocillin X was isolated as a light yellow solid as follows: melting point, 238–240 °C; High Resolution Mass Spectrum, found 288.0927 (calculated for $N_{18}H_{12}N_2O_2$, 288.0899). IRυ_{max} ($CHCl_3$, cm^{-1}): 3300, 2150 (CN), 1590, 1514, 1433; MS (EI) *m*/*z* (rel. int.), 371 (62), 289 (40), 214 (27), 195 (17), 156 (9); 1H NMR (DMSO), δ 10.28 (s, 2H), 7.7 ppm (d, 4H, J = 8.7) 6.9 (d, 4H, J = 8.7), 7.05 (s, 2H). These values are in accord with those reported in the literature [16].

Discussion

In temperate climates, *P. chrysogenum* can be introduced indoors from several sources, including food and decaying vegetation [9] as well as new growth inside the building. *Penicillium chrysogenum* was the most common of 28 species of *Penicillium* isolated from moldy building materials by four large commercial laboratories in the US and Canada (''common'', ''frequent'', ''infrequent'', ''rare''). It was the only *Penicillium* species that was common on all six materials considered, namely insulation, gypsum wallboard, wood, manufactured wood, ceiling tiles and textiles [3]. When isolated from moldy gypsum wallboard samples collected from six buildings with different types of moisture failures, it was one of only a few species of *Penicillium* common under all circumstances [1].

Two groups of *P. chrysogenum* strains were included in the study. The first included 13 strains studied by Scott et al. [9] as deposited in DAOM. This included isolates from indoor environments, soil, paper, and food products. The second group comprised 17 strains, mainly from indoor air samples (i.e. from RCS or Andersen samplers) and moldy building materials (Table 1). The principal metabolites found included roquefortine C, meleagrin, penicillin G and xanthocillin X. The latter compound was not detected in any of the culture collection strains and two of these strains failed to produce any compounds under the conditions we used. Otherwise, metabolite yields were much attenuated in the strains from the culture collection compared to the strains collected in 2003 and 2004.

Most strains tested were good producers of penicillin G and roquefortine C. Meleagrin was a more important metabolite of this fungus compared to previous studies. Otherwise, two strong groups of strains differentiated by xanthocillin X accumulation were found. Xanthocillin X was discovered in the mid 1940s from *P. chrysogenum* and commercially produced as an antibiotic. It is a more potent contact allergen than penicillin [17], and its other biological properties are still being investigated [18]. Although a much smaller number of buildings were involved compared to the survey by Scott et al. (16 vs .∼200), we also found that the large majority of our strains were in clade 4, with a modest number of strains in Clade 1. We have provided evidence that the dominance of clade 4 in buildings is widespread, noting that our present strains come from BC, Alberta, Saskatchewan as well as northern, central and eastern Ontario (Table 1). There was no evidence of chemotype differences between the two clades, but the number of clade 1 strains only represented ∼10% of the total.

An additional objective of this research was to determine the toxigenic potential of the dominant molecular species of *P. chrysogenum* associated with building materials (clade 4). It is known that one or more the toxins from fungi growing in building materials can accumulate in house dust [19] and have been found in high volume air samples [20]. With this information, in vivo lung toxicity tests have been completed on one of the metabolites discussed, roquefortine C. We found that mice intratracheally exposed to roquefortine C, TNF-ά and MIP-2 levels were significantly elevated in animals receiving >5.0 nM/g BW treatment group in a dose and time dependent fashion [21]. The latter effect was common after low dose exposure to the fungal metabolites thus far. This suggests that aspects of the C–X–C pathway are important in the non allergic effects associated with mold in damp housing [21, 22].

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