NOTES

Production of Penicillic Acid and Patulin by an Atypical
Penicillium roqueforti Isolate†

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Simultaneous production of penicillic acid and patulin by an atypical strain of Penicillium roqueforti isolated from cheddar cheese is reported. Mycotoxin production was confirmed by thin-layer and gas-liquid chromatography and by ultraviolet, infrared, and mass spectral analyses. Culture extracts were toxic to Bacillus megaterium and chicken embryos. Commercial strains of P. roqueforti used in production of blue-veined cheeses were shown not to produce penicillic acid and patulin.

In a recent study of the mold flora of cheddar cheese, we reported that a number of mold isolates were capable of producing patulin and penicillic acid in yeast-extract sucrose broth (1). In subsequent work with one of the isolates designated M-247, it was observed that this organism appeared to be capable of producing penicillic acid and patulin simultaneously. Preliminary examination of the culture indicated that the organism appeared to be an atypical Penicillium roqueforti. Since we were unaware of previous reports of the simultaneous production of penicillic acid and patulin by P. roqueforti, the purpose of the work reported here was to verify the identity of the organism, confirm the presence of penicillic acid and patulin in culture extracts, and to determine whether commercial cultures of P. roqueforti might also possess this ability.

Upon close examination, it was found that the culture clearly belonged in the Asymmetrica section of the genus Penicillium. The correct placement of the species, however, was somewhat in doubt, even though the classification systems of Thom (17) and Raper and Thom (12) were consulted. Initial attempts to identify the isolate to species level, using the key of Raper and Thom (12), led to uncertainty as to whether it was P. cyclopium or P. roqueforti. Culture M-247 differed from P. cyclopium in that the colony color was dark green rather than the typical blue-green, the mycelial mat was very thin, and the culture lacked the typical strong Actinomycetes-like odor. Reverse color of the isolate was a pale green, not the dark greenish black normally associated with P. roqueforti, and the typical arachnoid margins of P. roqueforti were not produced. The conidia were smooth and globose, never deviating toward subglobose or elliptical, as is common in P. cyclopium. Conidial chains were produced in long columnar masses that clung together, often even in a wet mount. Conidiophores, branches, and metulae were conspicuously encrusted when grown in Czapek or malt agar (Fig. 1). These echinulations were very coarse and knobby, much more similar to P. roqueforti than to P. cyclopium. When we referred to earlier classification keys, this organism showed a striking similarity to Thom's description of P. asperulum Bainier, which he listed immediately after the species he believed to be nearly inseparable from P. roqueforti (17). In their 1949 manual, Raper and Thom assigned P. asperulum as "probably some member of the P. roqueforti series" (12). From these observations, the organism was identified as an atypical P. roqueforti.

To study toxin production the mold was grown on 900 ml of 2% yeast extract–15% sucrose broth in Fernbach flasks at 12°C for 14 days. The cultures were macerated in a blender, filtered to remove particulate material, and extracted with two equal 900-ml volumes of chloroform. The extracts were then concentrated in vacuo, transferred to vials, and dried in a stream of nitrogen. Penicillic acid and patulin fractions of the extract were separated, identified by comparison to standards, and collected by using preparative thin-layer chromatography plates (20 by 20 cm;
Silica Gel G-HR, Brinkmann Instruments Inc.) developed in chloroform–ethyl acetate–90% formic acid (60:30:10) (CEF). The bands corresponding to the $R_f$ of penicillic acid and patulin were previously determined by derivatization with ammonia and phenylhydrazine (14). These bands were collected, and the respective mycotoxin was eluted from the silica gel by refluxing with chloroform for 1 to 2 h in a Mini-Soxhlet apparatus. The fractions were checked by thin-
layer chromatography to confirm the presence of only one of the desired metabolites in each fraction; if the fraction contained both compounds, preparative thin-layer chromatography was repeated until a clean fraction was obtained. The resulting fractions, designated as PA (penicillic acid) and PAT (patulin), were used to confirm the identity of the compounds by determining the various spectral and chromatographic properties of the fractions.

On thin-layer chromatography analysis, the PA fraction had an Rf of 0.73, and the PAT fraction had an Rf of 0.64 (Silica Gel G-HR plates, 0.25 mm thick, developed in CEF). When derivatized with ammonia, phenylhydrazine, and p-anisaldehyde (14), the Rf values and color reactions were identical to those of standard PA and PAT. Ultraviolet absorption spectra (Beckman model 25) gave results for the PA (241 nm) and PAT (276 nm) fractions identical to those for the PA and PAT standards. The infrared spectra (Beckman model IR-5A) of the two fractions corresponded to those of the two mycotoxin standard compounds, with absorption bands for the PA fraction at 5.7 and 6.1 cm⁻¹ and for the PAT fraction at 5.7, 6.0, and 6.1 cm⁻¹. Gas-liquid chromatography (model 1740 Varian Aerograph with model 30 Varian Aerograph recorder; column conditions: stainless steel, 1.7 m by 3.2 mm OD, 3.0% OV-7 and 1.5% OV-22 on 80- to 100-mesh Chromosorb G, Varian Aerograph; conditioned at 250°C for 24 h; N2 carrier gas flow of 25 ml/min; injector block and detector temperatures of 250°C; temperature program of 160 to 250°C in increments of 6°C/min; electrometer range, 10⁻¹⁰ A/mV) was done, comparing both the nonderivatized and the trimethylsilyl-derivatized ('Tri-Sil-BSA- [N,O-Bis-(trimethylsilyl)-acetamide]; Pierce Chemical Co., Rockford, Ill.) forms of the two fractions to the same forms of the mycotoxin standards (11). Retention times for the PA standard were 6.3 and 5.4 min, and those for the PAT standard were 8.7 and 7.3 min (nonderivatized and derivatized, respectively). The PA and PAT fractions had similar retention times. Finally, the mass spectral patterns of the PA and PAT fractions were compared with authentic PA and PAT standards by using an AE1 MS 50 high-resolution mass spectrometer (source temperature, 200°C; ionizing voltage, 70 eV). It was found that the mass spectral peaks of the fragmentation patterns of the fractions matched those of the authentic compounds. The values presented are in agreement with those reported in the literature (6, 10, 11, 14–16, 18, 20) and confirm that both penicillic acid and patulin were produced by the culture. The culture extract was inhibitory to Bacillus megaterium and toxic to chicken embryos. Both penicillic acid and patulin inhibit the growth of B. megaterium (7), and both are toxic to chicken embryos, in which the mean lethal dose of penicillic acid is 0.85 mg/egg via air sac (3) and that of patulin is 66.7 µg/egg via air sac (4).

P. roqueforti, the essential fungus used in the manufacture of Roquefort and other varieties of blue cheeses, has been shown to produce other toxic compounds. These include PR toxin (19) and roquefortine, a neurotoxic alkaloid, which has been detected in blue cheese samples from seven different countries (13). Orth (9) reported finding PR-toxin-producing P. roqueforti isolates in nut ice cream, a dessert cream, and on a red wine bottle cork. However, the significance of these toxins in terms of human health is unclear, particularly because blue-veined cheeses have been consumed for centuries without apparent ill effects.

The potential carcinogenic nature of penicillic acid and patulin (5) possibly makes them a cause for concern. However, work that we have reported elsewhere with M-247 has shown that these toxins may not be produced in proteinaceous substrates, such as cheese (Olivigni and Bullerman, J. Food Sci., in press). Also, it is possible that commercial cultures of P. roqueforti used in blue cheese production do not produce penicillic acid and patulin. This latter point was studied in our laboratory, using cultures obtained from commercial suppliers of P. roqueforti cultures (Dairyland Food Laboratories Inc., Waukesha, Wis., and Laboratorium Visby, Tønder, Denmark) and cultures isolated directly from blue-veined cheeses. Three commercial strains of P. roqueforti and seven isolates obtained from domestic and imported blue, Roquefort, and Gorgonzola cheeses were tested for their ability to produce penicillic acid and patulin. Each culture was grown on 50 ml each of 2% yeast extract–15% sucrose and potato-dextrose (8) broth in 250-ml Erlenmeyer flasks at 12°C for 14 days. In addition, culture M-247 and a patulin-producing strain of P. patulum, also isolated from cheese, were grown under the same conditions and used as controls. All cultures were separated into mold mat and broth fractions, and each was extracted separately. The yeast extract-sucrose broth and corresponding mold mats were each extracted with two 50-ml volumes of chloroform. The potato-dextrose broth and corresponding mold mats were each extracted with two 50-ml volumes of ethyl acetate. Samples were concentrated and examined by thin-layer chromatography, as previously described. All commercial cultures and isolates of P. roqueforti obtained from the blue-veined cheeses were found negative for both penicillic...
acid and patulin. However, control culture M-247 produced detectable amounts of patulin (75 μg/ml) on potato-dextrose broth and both penicillic acid and patulin (100 and 35 μg/ml, respectively) on yeast extract-sucrose broth. P. patulum likewise produced detectable amounts of patulin in both potato-dextrose (2.8 mg/ml) and yeast extract-sucrose (1.4 mg/ml) broths. Thus, whereas the known toxin-producing cultures produced easily detectable amounts of penicillic acid and patulin under the conditions of this test, the commercial strains of P. roqueforti did not.

Several species of Penicillium and Aspergillus have been reported to produce either patulin or penicillic acid (2). Ciegler (2) previously listed P. roqueforti as capable of producing penicillic acid, but no reference has been found associating it with patulin production. Also, to our knowledge this is the first report of any isolate producing both mycotoxins.

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LITERATURE CITED


