Biochemical Characterization of Ochratoxin A-Producing Strains of the Genus *Penicillium*

THOMAS OUSTENFELD LARSEN,* ANNE SVENDSEN, AND JØRN SMEDSGAARD

BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Received 8 January 2001/Accepted 25 May 2001

In order to explore the biochemical scope of ochratoxin A-producing penicillia, we screened 48 *Penicillium verrucosum* isolates for the production of secondary metabolites. Fungal metabolites were analyzed by high-pressure liquid or gas chromatography coupled to diode array detection or mass spectrometry. The following metabolites were identified: ochratoxins A and B, citrinin, verrucolones, verrucines, anacines, sclerotigenin, lumpidin, fumiquinazolines, alantrypinones, dalldinin D, dipodazine, penigequinolines A and B, 2-pentanone, and 2-methyl-isoborneol. By use of average linking clustering based on binary (nonvolatile) metabolite data, the 48 isolates could be grouped into two large and clearly separated groups and a small outlying group of four non-ochratoxin-producing isolates. The largest group, containing 24 isolates, mainly originating from plant sources, included the type culture of *P. verrucosum*. These isolates produced ochratoxin A, verrucolones, citrinin, and verrucines and had a characteristic dark brown reverse color on yeast extract-sucrose agar medium. Almost all of a group of 20 isolates mainly originating from cheese and meat products had a pale cream reverse color on yeast extract-sucrose agar medium and produced ochratoxin A, verrucolones, anacines, and sclerotigenin. This group included the former type culture of *P. nordicum*. We also found that *P. verrucosum* isolates and three *P. nordicum* isolates incorporated phenylalanine into verrucine and lumpidin metabolites, a finding which could explain why those isolates produced relatively lower levels of ochratoxins than did most isolates of *P. nordicum*.

Ochratoxin A is an important nephrotoxic and nephrocarcinogenic mycotoxin which, despite having been reported from numerous species, is produced only by a relatively small number of species in the genera *Aspergillus*, *Petromyces*, *Neopetromyces*, and *Penicillium* (8). Black aspergilli, such as *Aspergillus niger*, *A. carbonarius*, and *A. ochraceus*, are important ochratoxin A (compound 1; Fig. 1)-producing species in the genus *Penicillium*. We also found that *P. verrucosum* is the only ochratoxin-producing species in the genus *Penicillium*. However, both Pitt (21) and Frisvad and Filtenborg (6) retained two chemotypes within the species, chemotype II being the only type capable of producing citrinin (compound 2; Fig. 1).

A number of *P. verrucosum* metabolites, such as verrucolone (compound 3; Fig. 1) and related α-pyrones, e.g., PC-2 (compound 4; Fig. 1), have been isolated (15; L. Rahbæk, S. Sperry, J. C. Frisvad, and T. O. Larsen, submitted for publication) from both chemotypes. A major metabolite with a retention index and a UV spectrum very similar to those of anacine, a benzodiazepine metabolite first reported for *P. aurantiogriseum* (1), was found in *P. verrucosum* extracts. However, the UV spectra of the two compounds were slightly different, and recently the structures of verrucines A (compound 5; Fig. 1) and B and a revised structure of anacine (compound 6; Fig. 1) were described. All three metabolites are quinazolines rather than benzodiazepines (12). Some additional oxygenated analogs of the verrucines have also been isolated from *P. verrucosum* (T. O. Larsen, unpublished results). Furthermore, two other quinazolines, fumiquinazoline F (compound 7; Fig. 1) and the spirocyclic metabolite alantrypinone (compound 8; Fig. 1), have been isolated from a morphologically atypical isolate originally identified as *P. verrucosum* (17).}

During UV-guided screening for novel diketopiperazine-producing penicillia, Larsen et al. (T. O. Larsen, B. O. Petersen, and J. Ø. Duus, submitted for publication) discovered that some isolates of *P. verrucosum* produced the metabolite lumpidin (compound 9; Fig. 1), with a UV spectrum very similar to that of verrucofortrine (10). Furthermore, some isolates identified as *P. verrucosum* also produced the benzodiazepine sclerotigenin (compound 10; Fig. 1) (16). Since none of the sclerotigenin-producing isolates produced citrinin, the study...
suggested a sharper separation of the two chemotypes; however, only eight isolates were studied.

The objective of this study was to determine if any systematic differences in metabolite production could be established when a relatively large number of possible ochratoxin-producing *Penicillium* isolates were examined simultaneously.

**MATERIALS AND METHODS**

Fungal isolates (Table 2) were obtained from the IBT Culture Collection, BioCentrum-DTU, Technical University of Denmark. Isolates were inoculated in triplicate on CYA (20) and YES (5) and incubated at 25°C for 7 days in the dark. Procedures for microextraction of fungal secondary metabolites and analytical high-pressure liquid chromatography conditions were similar to those described in previous studies.

**TABLE 1. Various species concepts for *P. viridicatum*, *P. verrucosum*, *P. nordicum*, *P. mediolanense*, and *P. casei***

<table>
<thead>
<tr>
<th>Original species named by:</th>
<th>Original name for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>Raper and Thom (23)</td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>Ciegler et al. (2)</td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>Pitt (20)</td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>Frisvad and Filtenborg (5)</td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>Pitt (21)</td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>Frisvad and Filtenborg (6)</td>
<td><em>P. viridicatum</em></td>
</tr>
<tr>
<td>This study</td>
<td><em>P. viridicatum</em></td>
</tr>
</tbody>
</table>
Retention indices (RI) for fungal metabolites were calculated as described by Frisvad (4).

Liquid chromatography (LC)-mass spectrometry (MS) analyses (Table 2) were performed with an HP-1100 system, an HP-1100 diode array detector, and an HP-1100 LC/mass selective detector coupled in series using atmospheric pressure chemical ionization. A Hypersil BDS C18 column (4 by 100 mm) with 3-μm particles was used for separation. A linear gradient of H2O-CH3CN (both containing 500 μl of formic acid/liter) was used; the gradient was changed from 10% CH3CN to 100% over 30 min and then was maintained at 100% CH3CN for 5 min before a return to starting conditions. The flow rate was 0.5 ml/min.

Volatile metabolites from 14 isolates (Table 2) were collected by diffusive sampling and analyzed by gas chromatography (GC)-MS as described by Larsen (11).

Cluster analysis of qualitative metabolite data (0/1 data) was performed using the software package NTSYS version 2.0 (Exeter Software, Setauket, N.Y.). The input data consisted of a 48-object (fungal isolates) and a 13-variable (biosyn-
The 24 isolates in cluster A (Fig. 1) were grouped on the basis of the production of several of the following metabolites (number of isolates in parentheses): ochratoxin A (17), verrucolones (19), anacines (19), metabolite I (16), sclerotigenin (11), and lumpidin (3). Included in subgroup B2 (Fig. 1) were IBT 12801 (=NRRL 3711) studied by Ciegler et al. (2); IBT 12808 (=NRRL 5574) studied by Ciegler et al. (2), Pitt (21), and Frisvad and Filtenborg (6); and the former type culture of *P. nordicum* (IBT 13508). The UV data indicated at least four metabolites in the anacine series. LC-MS indicated two protonated anacine isomers (m/z 343) and two possible protonated hydroxylated isomers (m/z 359), as seen for the verrucines (Larsen, unpublished results).

A subgroup (B1; Fig. 1) of three isolates (IBT 6573, IBT 12806 (=NRRL 1161), and IBT 13943) can be seen in cluster B. These isolates were grouped together since they were the only isolates producing lumpidin as a major component. The former type culture of *P. mediolanense* (IBT 5066) was separated (B3; Fig. 1) from the rest of the isolates in cluster B as the only isolate not producing ochratoxins and metabolite I. All isolates in subgroup B2 (Fig. 1) were light cream or dull yellow on the reverse on YES agar plates.

A small group of 4 isolates (cluster C; Fig. 1) clearly appeared as an outgroup within our set of 48 isolates. The major metabolites produced by these isolates were fumiquinazoline F and anaptenones, together with verrucolones, anacines, and dipodazine. Three out of the four isolates produced daldinin D, while penigequinoles A and B were detected only from one isolate (IBT 5891). These four isolates had the most orange-yellow reverse on YES agar plates of all the isolates (Table 2). None of the four isolates produced ochratoxins or citrinin, as did cluster A and B isolates.

A large similarity in patterns of volatile metabolites could be seen from the GC-MS analyses (results not shown). The major volatile compounds of all the isolates were 2-pentanone and small alcohols, such as isobutanol and isopentanol. In addition, all of the isolates produced the moldy odorous monoterpene 2-methyl-isoborneol (compound 12; Fig. 1).

**DISCUSSION**

Ochratoxin A- and citrinin-producing isolates. The large group of 24 isolates that clustered with the type culture of *P. verrucosum* (cluster A; Fig. 1) fits very well with group II isolates of Ciegler et al. (2), Pitt (21), and Frisvad and Filtenborg (6). One isolate, IBT 5092 (=NRRL 5571), was included in group II in all three studies. We found that 79% of the isolates produced ochratoxin A and 83% produced citrinin, results which parallel the findings of Ciegler et al. (2). However, we did not detect ochratoxin A or citrinin production by the *P. verrucosum* type culture, probably because it has been in cultures for many years. Variation from normal is to be expected, as concluded by Pitt (21), who found that the type
culture did not produce citrinin. It is likely that this is the reason why Frisvad and Filtenborg (6) placed the type culture in their chemotype I rather than in their chemotype II. We found that the type culture of *P. verrucosum* produces verrucines, like 23 out of 24 isolates in cluster A, and verruculones (Fig. 1), but never anacines or sclerotigenin, which are typical of the isolates grouping together with the former type culture of *P. nordicum* in cluster B (Fig. 1). Therefore, our results clearly place the type culture of *P. verrucosum* in cluster A.

**Ochratoxin A- but non-citrinin-producing isolates.** The large group of 20 isolates in cluster B corresponds to the group III isolates described by Ciegler et al. (2) and Pitt (21) and to chemotype I of Frisvad and Filtenborg (6) and includes IBT 12808, which was investigated in all three studies. None of these isolates produced citrinin or verrucines unique to cluster A isolates; instead, all 20 isolates produced anacines, 11 isolates produced sclerotigenin, and 3 isolates produced lumpidin, metabolites not produced by any of the isolates in cluster A.

The present work is thus the first to demonstrate that non-citrinin-producing isolates of *P. verrucosum* produce metabolites not produced by citrinin-producing isolates (Fig. 1) and hence may be clearly distinguishable. Cluster B includes the former type culture of *P. nordicum* (IBT 13308), together with isolates originating from meat and cheese. *P. nordicum* was first described by Dragoni and Cantoni (3), with the Latin diagnosis required to validate the name given by Ramírez (22).

The three isolates (IBT 6537, IBT 12806, and IBT 13943) in subgroup B1 (Fig. 1) are the only isolates producing lumpidin. In addition to lumpidin, they also produce metabolites typical of subgroup B2 isolates (verruculones, anacines, and ochratoxin A but not sclerotigenin). These three isolates did not have the typical pale cream reverse color of *P. nordicum* on YES agar but instead were more yellow (Table 2). Ciegler et al. (2) also included IBT 12806 (as NRRL 1161) among their *P. viridicatum* III isolates, with the remarks that the isolate also had features in common with their type II isolates. Further studies are needed to establish whether lumpidin-producing isolates belong to a separate species or should remain as a chemotype within cluster B isolates.

**Non-ochratoxin A-producing isolates.** The distinct cluster C (Fig. 1) represents a group of four biochemically unique isolates that produce alantrypinones and fumiquinazoline F as the major metabolites. These four isolates also could be separated from the rest of the isolates due to their pronounced orangeyellow reverse color on YES agar (Table 2). However, these isolates also produce metabolites such as the verruculones, anacines, 2-pentanone, and 2-methyl-isoborneol, as do the other studied isolates, indicating a close relationship between cluster C isolates and cluster A and B isolates. As for subgroup B1 (Fig. 1) isolates, further studies are needed to correctly identify and classify these four cluster C isolates and to determine whether they are capable of producing ochratoxins under growth conditions other than those used here.

**Production of volatile metabolites.** The very similar profiles of volatile metabolites produced by representative isolates (Table 2) of clusters A to C suggest that they are closely related, despite the differences in the production of nonvolatile metabolites. These results agree to some extent with those of Ciegler et al. (2), who also found 2-pentanone to be the major volatile compound of isolates of *P. viridicatum* II. Our findings thus are concordant with the results found for the series Viridi-cata, where closely related species such as *P. aurantiogriseum*, *P. polonicum*, *P. tricolor*, and *P. cyclopium* have been found to produce almost identical profiles of volatile sesquiterpenes (13, 14).

**Competitive biosynthesis involving phenylalanine.** In general, isolates in the *P. nordicum* group produced much larger amounts of ochratoxins than did isolates in the *P. verrucosum* group. One reason for this difference could be that phenylalanine is used in the biosynthesis of both ochratoxins and verrucines by isolates in the *P. verrucosum* group but in the biosynthesis of only ochratoxins by the *P. nordicum* group of isolates (Fig. 2). Instead of verrucines, isolates in the *P. nordi-cum* group produce anacines, which are very similar quinazoline metabolites that incorporate leucine instead of phenylalanine.

This hypothesis, competitive biosynthesis between different pathways that use phenylalanine, is supported by the fact that the three isolates that produce lumpidin (B1; Fig. 1) also are relatively poor ochratoxin producers. Another likely reason for lower ochratoxin A production by isolates in the *P. verrucosum* group is the incorporation of the similar basic isocoumarin moiety into both ochratoxin A and citrinin (Fig. 2).

**Chemosystematic and ecological significance.** In conclusion, 44 of the 48 isolates that we examined can be separated into two large groups (A and B) of ochratoxin A-producing isolates (Fig. 1) by cluster analysis based on metabolite production. Our results are consistent with those of Ciegler et al. (2). However, they did not describe their *P. viridicatum* II and III as new subspecies or varieties of *P. viridicatum* due to the lack of distinctive morphological characteristics.

We suggest that the two large groups represent the species *P. verrucosum* and *P. nordicum* and that both species belong to the series Verrocus, subgenus *Penicillium*. Despite the facts that both *P. verrucosum* and *P. nordicum* are slowly growing species, with very similar colony diameters on many media, and that both have rough stipes, they can be clearly distinguished from each other by use of the following criteria: (i) these
species produce different secondary metabolite profiles (Fig. 1); (ii) under many laboratory conditions, P. nordicum isolates produce more ochratoxin than P. verrucosum isolates; (iii) P. verrucosum cultures commonly have a diagnostic dark brown reverse color on YES agar, whereas P. nordicum cultures have a pale, creamy, or dull yellow reverse color on YES agar (in the present study, two nonbrown P. verrucosum isolates both produced citrinin, a metabolite that can be easily detected by thin-layer chromatography [24]); and (iv) all meat-derived ochratoxin A-producing isolates that we examined were P. nordicum, whereas all isolates originating from plant-derived material were P. verrucosum (Table 2), suggesting that the two species usually occupy different ecological niches.

Assigning ochratoxin-producing penicillia to either P. verrucosum or P. nordicum is very important because it emphasizes that despite sharing the ability to produce ochratoxins, these groups of fungi are biosynthetically and ecologically very different. Future work should result in more detailed insight into the biotic and abiotic factors that influence and regulate the biosynthesis of ochratoxins and other secondary metabolites produced by P. verrucosum and P. nordicum. Such studies might also lead to a better understanding of the nature of the apparently different ecological origins of P. verrucosum and P. nordicum.

ACKNOWLEDGMENTS

This research was supported in part by the Advanced Center for Food Research, the Danish Dairy Foundation (Danish Dairy Board), the Danish Research and Development Program for Food Technology, and the Danish Research Councils (grant 9800555). We thank Agilent Technologies, Birkerød, Denmark, for the use of the HP-1100 LC/mass selective detector, Hanne Jakobsen for performing the LC-MS analyses, Amira Azar and Trine Camilla Rasted for the HP-1100 LC/mass selective detector, Hanne Jakobsen for performing the analyses, and the Danish Research Councils (grant 9800555).

REFERENCES