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Molecular Identification of Species from the *Penicillium roqueforti* Group Associated with Spoiled Animal Feed

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The *Penicillium roqueforti* group has recently been split into three species, *P. roqueforti*, *Penicillium carneum*, and *Penicillium paneum*, on the basis of differences in ribosomal DNA sequences and secondary metabolite profiles. We reevaluated the taxonomic identity of 52 livestock feed isolates from Sweden, previously identified by morphology as *P. roqueforti*, by comparing the sequences of the ribosomal internal transcribed spacer region. Identities were confirmed with random amplified polymorphic DNA analysis and secondary metabolite profiles. Of these isolates, 48 were *P. roqueforti*, 2 were *P. paneum*, and 2 were *Penicillium expansum*. No *P. carneum* isolates were found. The three species produce different mycotoxins, but no obvious relationship between mold and animal disease was detected, based on medical records. *P. roqueforti* appears to dominate in silage, but the ecological and toxicological importance of *P. carneum* and *P. paneum* as feed spoilage fungi is not clear. This is the first report of *P. expansum* in silage.

A central issue in the field of feed quality and storage is the problem of mold spoilage. Fungal growth reduces nutritional value and may result in the production of mycotoxins and allergenic spores. One way of preserving grass forage is ensiling, in which organic acids produced by lactic acid bacteria and low oxygen pressure prevent growth of spoilage molds and bacteria. However, nonuniform distribution of acids or failure to maintain a low oxygen pressure, especially when breaking silos and big bales for feedout, often induces the growth of microaerophilic acid-tolerant molds. Members of the genus *Penicillium* are commonly found in feedstuffs in temperate climates. *Penicillium roqueforti*, which can grow on organic acids (7), is the dominant fungus in most silage samples (1, 16, 19, 24). Animal health disorders are correlated with the production of toxic metabolites in vitro (6, 14, 26). Recently, Auerbach et al. (1) reported that 21 of 24 visibly moldy silage samples contained roquefortine C. This neurotoxic (29) mycotoxin as well as the mutagenic (23, 28) PR toxin, both produced by *P. roqueforti*, are believed to be involved in disease symptoms observed in farm animals, i.e., extensive paralysis of cows (12) and bovine abortion and placental retention (26), respectively.

P. roqueforti was recently split into the three species, *P. roqueforti*, *Penicillium carneum*, and *Penicillium paneum*, (collectively referred to as the *P. roqueforti* group) based on ribosomal DNA sequence comparison, random amplified polymorphic DNA (RAPD) profiles, and secondary metabolite profiles (2). These three species synthesize different mycotoxins. All three produce roquefortine C, only *P. roqueforti* produces PR toxin, and both *P. carneum* and *P. paneum* produce patulin, which is mutagenic, immunotoxic, and neurotoxic (5, 8). PR toxin is the most acutely toxic metabolite produced, with 50% lethal dose values in mice ranging from 1 to 5.8 mg kg of body weight^{-1} (intraperitoneally [IP]) (5). The equivalent 50% lethal dose values for roquefortine C and patulin are 20 and 5 mg kg of body weight^{-1} (IP), respectively (5, 25). All three *Penicillium* species can grow on 0.5% acetic acid (2) and have similar microaerophilic capacity with regard to their ability to grow at low oxygen and high carbon dioxide pressures (20).

Our objectives in this study were: (i) to determine the natural occurrence and/or distribution of *P. roqueforti*, *P. carneum*, and *P. paneum* in animal feed and (ii) to relate, when possible, mold identity to animal disease. Our results are the first indication of the relative importance, as feed spoilage organisms within the *P. roqueforti* group, of the newly defined species *P. carneum* and *P. paneum*.

MATERIALS AND METHODS

Fungal isolates and isolation method. The National Veterinary Institute (SVA), Uppsala, Sweden, supplied strains isolated between 1988 and 1998 from feed suspected to be the cause of various diseases in livestock. Isolates were picked from either dichloran-glycerol (DG18) (Oxoid, Basingstoke, Hampshire, England) or dichloran rose bengal chloramphenicol (DRBC) (Oxoid) agar plates after incubation at 25°C for 5 to 6 days. Isolates identified as *P. roqueforti* by microscopy were stored on Czapek yeast (autolysate) extract (CYA) agar (Oxoid) slants at 4°C at the SVA. Type strains of *P. roqueforti* (IBT 6754) (Institut for Bioteknologi Culture Collection, Technical University of Denmark [DTU], Lyngby, Denmark), *P. carneum* (IBT 6884), and *P. paneum* (IBT 12407) were supplied by Jens C. Frisvad, Department of Biotechnology, DTU.

Of the 48 *P. roqueforti* isolates, 29 strains were obtained from grass silage and 3 isolates each from ensiled hard-pressed beet fibers, ensiled grain, and whey. The remaining 10 *P. roqueforti* strains were isolated from various feeds or came from unknown sources. *P. paneum* was found in grass silage and hard-pressed beet fibers, while *Penicillium expansum* was only found in grass silage.

Growth media and conditions. All isolates were grown on DG18 agar (Oxoid) for 7 days at 25°C in the dark before DNA extraction. For determination of tolerance to acetic acid, isolates were inoculated on malt extract agar (Oxoid) with 0.5% (vol/vol) glacial acetic acid added after autoclaving (MEA-HAc) and incubated in the dark for 4 days at 25°C. For secondary metabolite profile analysis, selected isolates were cultured on CYA agar for 7 days at 25°C in the dark (Czapek broth was purchased from Oxoid, and yeast extract was purchased from Difco [Detroit, Mich.]) (22).

Genomic DNA extraction. DNA was extracted from pure cultures on DG18 by either of two methods. (i) Agar plugs were excised from the plates and DNA was extracted using Fast DNA Prep Kit H (BIO 101, Vista, Calif.). (ii) Spore suspensions were made by adding 500 μl of diluent (0.85 g NaCl, 0.1 g Tween 80, and 0.05 g of agar per 100 ml of H_2O) to the plates, followed by slow shaking. Then 50 μl was transferred to an Eppendorf tube, mixed with 50 μl of lysis solution (0.2 M NaOH, 0.2% sodium dodecyl sulfate), heated to 105°C for 30 min, and cooled on ice (18). Samples were neutralized with 1/20 volumes of 5 M potassium acetate (pH 5.5), followed by the addition of 2 volumes of 6 M guanidine thiocyanate (IBI Molecular Biology Certified, Kodak, Rochester, N.Y.), and debris was pelleted for 5 min. The supernatant was transferred to a new tube, 50 μl of silica mixture (2.5 g of silica in 10 ml of 4% Triton X-100

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TABLE 1. Sequence comparison of isolates[a]

Reference species and EMBL accession no.	No. of isolates	Sequence position relative to the 5′ end of the ITS5 primer												
		106	128	145	147	150	179	180	181	199	202	211	212	213
P. roqueforti X82358		A	A	T	T	A	–	C	C	A	C	A	A	C
	48	•	•	•	•	•	–	N[b]	•	•	•	•	•	•
	2	G	•	C	C	G	C	•	•	T	T	T	G	A
P. paneum X82360		A	C	C	C	G	–	C	C	A	A	T	G	A
	2	•	•	•	•	•	–	•	•	•	•	•	•	•
P. carneum X82359		A	A	T	C	A	–	T	C	A	C	A	A	C
	0	•	•	•	•	•	–	•	•	•	•	•	•	•

[a] Sequence comparison of relevant positions from the ITS1 region of previously published reference isolates (2) and 52 strains isolated from feed and tentatively identified as *P. roqueforti* by microscopy. Dashes (–) indicate deletions and bullets (•) indicate identical nucleotides.

[b] In two cases, position 180 was T and in one case A, while the remaining 45 positions had a C residue.

[silica, 0.5 to 10 μm, was purchased from Sigma Chemical Co., St. Louis, Mo.) was added, and the sample was incubated for 5 min at 55°C. After cooling on ice, the silica was pelleted, washed twice in cold 80% ethanol, and dried in a heating block for 1 to 2 min. The silica was resuspended in 50 μl of distilled water, heated for 3 min at 55°C, cooled on ice, and pelleted. The DNA-containing supernatant was transferred to a new tube and stored at −20°C.

PCR conditions. PCR fragments for sequence analysis were generated using primers ITS4 and ITS5 covering the internal transcribed spacer 1 (ITS1), 5.8S, and ITS2 region of the ribosomal DNA (31). For each 50-μl reaction, a reaction mixture was prepared containing 10 ng of genomic DNA, 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphates (Pharmacia Biotech, Uppsala, Sweden), 0.25% Tween 20, 10% dimethyl sulfoxide, 0.3 μM (each) of ITS4 and ITS5, and 2.5 U of *Taq* DNA polymerase (Amersham Life Science, Buckinghamshire, United Kingdom). Reactions were run on a capillary thermocycler (Rapidcycler; Idaho Technology, Idaho Falls, Idaho) with an initial denaturation of 30 s at 94°C, followed by 30 cycles of 94°C for 15 s, 53°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 5 min.

Purification of PCR products. Aliquots of the PCR were electrophoresed on 1.5% (wt/vol) agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA), pH 7.7. DNA fragments of 600 bp each were eluted from the gel and then melted at 55°C with 3 volumes of 6 M guanidine thiocyanate. Twenty to thirty microliters of silica mixture (see above) was added, and incubation was continued for 5 min at 55°C. After cooling on ice, the silica was pelleted, washed twice in cold 80% ethanol, and dried in a heating block (55°C) for 1 to 2 min. The PCR fragments were eluted from the silica in 20 to 25 μl of distilled water for 3 min at 55°C. Finally, 5 μl of the eluent was loaded onto a new gel to estimate the DNA concentration.

DNA sequencing. We used an ABI 377 automatic sequencer (Perkin-Elmer Cetus, Branchburg, N.J.) and the Thermo Sequenase dye terminator cycle sequencing premixture kit (Amersham Life Science). For each 10-μl reaction, we used 6 pmol of either ITS4 or ITS5 and 0.5 to 1.0 μg of the purified PCR fragment. Sequences were evaluated with ABI EditView software (Perkin-Elmer Cetus) and compared with previously published sequences of *P. roqueforti*, *P. carneum*, and *P. paneum* (2).

RAPD fingerprinting. We made RAPD fingerprints of selected isolates as described by Boysen et al. (2) with the universal primers NS2 or NS7 (31). Amplification was performed on a PCT 1196 thermocycler (MJ Research, Watertown, Mass.) with an initial denaturation for 5 min at 94°C and was followed by 40 cycles of 94°C for 5 s, 40°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. The type strains of *P. roqueforti*, *P. carneum*, and *P. paneum* were used as controls.

Secondary metabolite profiles. Secondary metabolite profiles from selected isolates were determined by thin-layer chromatography (TLC) (11). From pure cultures grown on CYA agar (Oxoid) for 7 days at 25°C in the dark, metabolites were transferred to the TLC plate (Merck, Darmstadt, Germany) by consecutively placing three agar plugs per culture, medium side down, for 30 s on each spot. This procedure was repeated on the opposite side of the plate. One side was eluted with chloroform-acetone-isopropanol (85:15:20) and the other side with toluene–ethyl acetate–90% formic acid (5:4:1). Standards of griseofulvin (Merck), patulin, PR toxin, and roquefortine C (Sigma) and extracts from type strains of *P. roqueforti*, *P. carneum*, and *P. paneum* were run in parallel. Results were evaluated under UV light (254 and 365 nm) without spraying. Metabolite band patterns were related to those of the standards.

RESULTS

All isolates were identified as *P. roqueforti* based on morphological characters at the SVA. Of 34 samples with recorded mold CFU levels, 13 samples had values exceeding 10^4 CFU g^{-1}, with 5 of these being greater than 10^7 CFU g^{-1}. In most cases, only one species was found.

The sequences of the ITS1 and 5.8S regions and parts of the ITS2 region of 52 isolates were compared to previously published sequences of *P. roqueforti*, *P. carneum*, and *P. paneum* (Table 1). Forty-five isolates had sequences identical to *P. roqueforti*, while three differed at position 180 with either a T or an A replacing the previously reported C (2). The RAPD profiles of these three isolates were similar to those previously published for *P. roqueforti* (2) (Fig. 1a and b). The secondary metabolite profiles were similar to those of the *P. roqueforti* type strain (Fig. 2). Two isolates had sequences identical to *P. paneum*, and two isolates had sequences that differed from those of all members of the *P. roqueforti* group. No *P. carneum* isolates were found among the 52 isolates. The identity of the two *P. paneum* isolates was confirmed by secondary metabolite profiles (Fig. 2) and RAPD analysis (Fig. 1a and b) (2). In a blind test, the SVA included one previously identified isolate of *P. roqueforti* and two previously identified isolates of *P. carneum* among the feed isolates. Both DNA sequence analysis and RAPD fingerprinting procedures correctly identified all three isolates (data not shown).

We also examined the RAPD and secondary metabolite profiles of the two isolates with unknown sequences to determine if they belonged to the *P. roqueforti* group. Neither RAPD analysis (Fig. 1a and b) nor secondary metabolite profiles (Fig. 2) were consistent with the inclusion of those strains in the *P. roqueforti* group. Based on morphology (e.g., 3.0- to 3.5-μm conidia and smooth-walled stipes) and physiology (e.g., acid production on creatine sucrose agar), these strains are now classified as *P. expansum*. Roquefortine C was produced by *P. expansum* and all isolates produced a number of secondary metabolites (Fig. 2). All 52 isolates were cultured on MEA-HAc, and all but the two *P. expansum* strains could grow on 0.5% (vol/vol) acetic acid.

Among the 28 *P. roqueforti* isolates associated with diseased animals, 20 were obtained from feed samples from animals with bovine mastitis, 3 were associated with high or increased rates of mortality, 3 were from animals with fertility problems, and 2 were from animals with general health problems. Both *P. paneum* isolates came from feed associated with animals with mastitis or general health problems. The remaining isolates came from feed samples without any record of associated animal disease.

DISCUSSION

We reidentified 52 animal feed isolates of *P. roqueforti* using ITS sequence comparison, RAPD analysis, and secondary me-

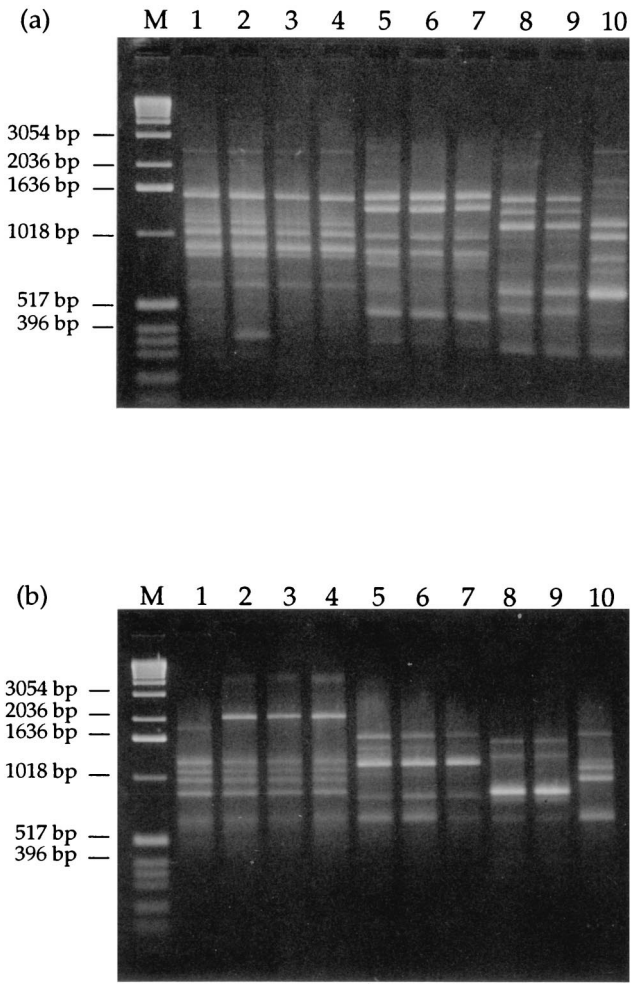

FIG. 1. RAPD fingerprinting using either NS2 (a) or NS7 (b) as a primer. Lanes: 1, *P. roqueforti* SVA 3631/1996, having a T in position 180 of the ITS1 region; 2, *P. roqueforti* SVA 8176/1995, isolate 5 (A in position 180); 3, *P. roqueforti* SVA 2986/1992 (T in position 180); 4, *P. roqueforti* type strain (IBT 6754); 5, *P. paneum* SVA 494/1990; 6, *P. paneum* SVA 7023/1994, isolate 2; 7, *P. paneum* type strain (IBT 12407); 8, *P. expansum* SVA 2294/1994, isolate 5; 9, *P. expansum* SVA 6180/1994 isolate 1; 10, *P. carneum* type strain (IBT 6884); M, molecular weight marker (1-kb DNA ladder; GIBCO BRL, Gaithersburg, Md.).

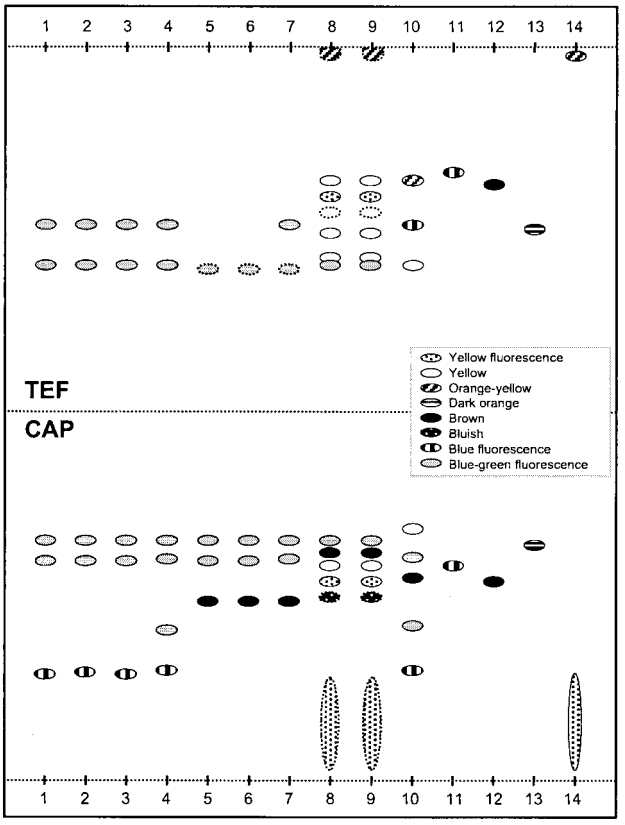

FIG. 2. Schematic illustration of secondary metabolite profiles. TLC plates were eluted in toluene-ethyl acetate-formic acid (TEF) and chloroform-acetone-isopropanol (CAP) and evaluated under UV light (365 nm). Lanes: 1, *P. roqueforti* SVA 3631/1996, having a T in position 180 of the ITS1 region; 2, *P. roqueforti* SVA 8176/1995, isolate 5 (A in position 180); 3, *P. roqueforti* SVA 2986/1992 (T in position 180); 4, *P. roqueforti* type strain (IBT 6754); 5, *P. paneum* SVA 494/1990; 6, *P. paneum* SVA 7023/1994, isolate 2; 7, *P. paneum* type strain (IBT 12407); 8, *P. expansum* SVA 2294/1994, isolate 5; 9, *P. expansum* SVA 6180/1994, isolate 1; 10, *P. carneum* type strain (IBT 6884). Lanes 11 to 14 are standards: griseofulvin (11), patulin (12), PR toxin (13), and roquefortine C (14). Dotted lines around spots indicate weak spots.

tabolite profiles as 48 *P. roqueforti*, 2 *P. paneum*, and 2 *P. expansum*. These results are consistent with previous results (J. C. Frisvad, personal communication), with *P. roqueforti* dominating and with limited occurrence of both *P. carneum* (not detected in our study) and *P. paneum*. Assuming that our sample is unbiased, then the frequency of *P. carneum* should be less than 6%.

Using a combination of methods for the identification of the isolates makes us confident that we have obtained correct strain identities. The taxonomic information recoverable from highly conserved DNA sequences, such as the ITS regions, can often give sufficient information (3), though it is not advisable to use these sequences as a sole criteria for characterization. However, even conserved regions have small variations, and in those cases we confirmed the identification by analyzing RAPD and secondary metabolite profile patterns. Though RAPD profiles are known to be difficult to reproduce (15), we have obtained consistent results using different DNA extraction methods and different thermocyclers in this and the initial work (2) for the RAPD analysis. The TLC analysis is a simple, fast screening method for analyzing secondary metabolites from mold (10). However, in our hands it was not sufficiently consistent for us to use it as the sole identification tool. Since the method is sensitive, e.g., to the origin of the yeast extract used in the substrates for secondary metabolite production, we could not be certain of an identification based only on individual metabolites. Instead, we used profiles of secondary metabolites, compared to those of reference strains, to complement the morphological and genetic information.

The fresh samples were not analyzed for mycotoxins, so possible relationships between mycotoxin and animal disease could not be evaluated critically. All three species of the *P. roqueforti* group can produce roquefortine C, the major mycotoxin found in moldy silage (1) or in culture broth of *P. roqueforti* isolates from silage (17, 30). Thus, mycotoxins could contribute to the animal diseases described in this study.

All three members of the *P. roqueforti* group have similar morphological and physiological characters, and for practical purposes Pitt and Hocking (21) consider the group a single species, even though some of the species can produce mycotoxins (e.g., PR toxin and patulin) that are more toxic than roquefortine C (2). No PR toxin or patulin production was

observed from the tested isolates of the *P. roqueforti* group (Fig. 2), but as mycotoxins can act synergistically, even low levels of several mycotoxins could be a health hazard (4, 9).

In general, good-quality silage feed contains less than 10^4 fungal CFU g of silage^{-1} (13), and acceptable feed should contain less than 10^5 CFU g^{-1} (24). To our knowledge, this is the first report of *P. expansum* being found in high numbers (>10^7 CFU g^{-1}) in silage. *P. expansum* is a common cause of apple rot and produces both roquefortine C and patulin (22). *P. expansum* is common in various fruits but is less common in stored or fresh foods (21). Like *P. roqueforti*, *P. expansum* has low oxygen requirements, is psychrophilic, and can grow at low water activity; the minimum water activity required for germination is 0.82 to 0.83 (21). Its ability to grow in acidic environments (e.g., apple, pH ~3.5) suggests that it may be a potential contaminant of silage. Indeed, preliminary results in our laboratory show that the two *P. expansum* isolates could grow on at least 0.3% (vol/vol) acetic acid (pH ~3) or 2% (wt/vol) lactic acid (pH ~2) in malt extract agar. Growth of *P. expansum* is inhibited by CO_2 levels of >15%, while growth of *P. roqueforti* is stimulated by CO_2 levels up to 15% and can occur even at 80% CO_2, provided the O_2 level is at least 4.2% (27).

This is the first report of the recovery of species within the *P. roqueforti* group from natural samples. We think that the closed and/or special microaerophilic environment and the organic acid substrate of silage favor growth of *P. roqueforti* and probably that of the entire *P. roqueforti* group. Further studies in other environments known to favor the *P. roqueforti* group (e.g., airtight stored grain or rye bread) are needed before concluding that *P. roqueforti* should be considered the primary or exclusive hazard of the three species.

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