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 pigs (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 Peni-
P. roqueforti X82358

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<th>No. of isolates</th>
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P. paneum X82360

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P. carneum X82359

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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.

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 10^6 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 stipites) and physiology (e.g., acid production on creatine sucrose agar), these strains are now classified as P. expansum. Roquefortin 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-
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 members of the *P. roqueforti* group can produce roquefortine C, the major myco- toxin 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 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 three of the species.

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**REFERENCES**


