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Production of Penicillin by Fungi Growing on Food Products: Identification of a Complete Penicillin Gene Cluster in *Penicillium griseofulvum* and a Truncated Cluster in *Penicillium verrucosum*

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Mycobiota growing on food is often beneficial for the ripening and development of the specific flavor characteristics of the product, but it can also be harmful due to the production of undesirable compounds such as mycotoxins or antibiotics. Some of the fungi most frequently isolated from fermented and cured meat products such as *Penicillium chrysogenum* and *Penicillium nalgiovense* are known penicillin producers; the latter has been shown to be able to produce penicillin when growing on the surface of meat products and secrete it to the medium. The presence of penicillin in food must be avoided, since it can lead to allergic reactions and the arising of penicillin resistance in human-pathogenic bacteria. In this article we describe a study of the penicillin production ability among fungi of the genus *Penicillium* that are used as starters for cheese and meat products or that are frequently isolated from food products. *Penicillium griseofulvum* was found to be a new penicillin producer and to have a penicillin gene cluster similar to that of *Penicillium chrysogenum*. No other species among the studied fungi were found to produce penicillin or to possess the penicillin biosynthetic genes, except *P. verrucosum*, which contains the *pcbAB* gene (as shown by hybridization and PCR cloning of fragments of the gene) but lacks *pcbC* and *penDE*. Antibacterial activities due to the production of secondary metabolites other than penicillin were observed in some fungi.

Many fungal species grow on food products, often contributing to their spoilage, as in fruits, vegetables, and other fresh or prepared products. However, in many instances the presence of fungi on some products does not have a deleterious effect and may even contribute to the ripening and development of important organoleptic characteristics typical of a particular food (17, 19). This is the case of fungi belonging to the genus *Penicillium*, which grow on cured meat products and on many kinds of cheese. Some of these fungi whose presence is beneficial for the product are nowadays used as starters, as a means to colonize homogeneously the cheese or meat piece, thus preventing the eventual colonization by undesirable fungi and bacteria. Some of the fungal species most widely used as starters are *Penicillium nalgiovense* and *Penicillium chrysogenum* for meat products, *Penicillium roqueforti* for Roquefort cheese, and *Penicillium camemberti* for Camembert cheese. Most fungi growing on food are producers of mycotoxins and antibiotics (28, 39, 46), and the presence of the compounds secreted by the fungus in the colonized food product has been reported on different occasions (9, 31). Laich et al. (25) showed that *P. nalgiovense* produces penicillin on the surface of a Spanish fermented sausage (fuet); the presence of the antibiotic can be detected in the outer layers of the sausages. The presence of penicillin in food is not desirable, as it can produce allergic reactions (18, 20, 29) and might eventually lead to the development of resistance phenomena in human resident bacteria, which could then transfer that genetic information to pathogenic bacteria, as resistance gene transfer between bacteria in the human colon has been shown to be a common phenomenon (42). The development of resistance phenomena in bacteria as a consequence of the ingestion of subtherapeutic levels of antibiotics has been reported in several cases in farm animals (1, 34).

Therefore, it is important to know whether fungi associated with food production are potential penicillin producers or not. The objective of the present work was to analyze the possible production of penicillin and to study the presence of the penicillin biosynthetic genes in fungi of the genus *Penicillium* growing on food products or used as starters for those products.

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MATERIALS AND METHODS

***Penicillium* strains.** Some of the fungal species were chosen for the present study because they are commonly used as commercial starters (*P. nalgiovense*, *P. chrysogenum*, *P. camemberti*, and *P. roqueforti*); the rest of the species were chosen because of their frequent isolation from cured meat products like cecina (a salted and smoked-cured beef meat product from north Spain) as part of the natural mycobiota growing on this product (F. Laich, F. Fierro, and J. F. Martín, unpublished results).

P. chrysogenum NRRL 1951; *P. nalgiovense* NRRL 911; *Penicillium griseofulvum* NRRL 2300, NRRL 2152, NRRL 989, NRRL 994, NRRL 991, and NRRL 992; *P. roqueforti* NRRL 849; *P. camemberti* NRRL 874, NRRL 877, NRRL 876 (ex-type of *Penicillium candidum*), and CECT 2267; *Penicillium verrucosum* NRRL 5573 and NRRL 5574; *Penicillium brevicompactum* NRRL 862; *Penicillium commune* NRRL 845; *Penicillium solitum* NRRL 937; *Penicillium crustosum* NRRL 972; *Penicillium expansum* NRRL 976; *Penicillium implicatum* NRRL 2061; *Penicillium hirsutum* NRRL 2032; *Penicillium aurantiogriseum* NRRL 971; *Penicillium viridicatum* NRRL 963; *Penicillium echinulatum* NRRL 1151; *Penicillium purpurogenum* ATCC MYA-38; and *Paecilomyces variotii* NRRL 1775 were obtained either from the ARS Culture Collection (Peoria, Ill.), the American Type Culture Collection (ATCC) (Manassas, Va.), or the Spanish type culture collection (CECT) (Valencia, Spain).

Other strains were isolated from cheese (inner part) and cecina (surface) as indicated by Motilva Casado et al. (36). For colony isolation, samples were plated on malt extract agar (MEA) (40) and incubated at 25°C for 7 days. The strains isolated were identified to species level according to the method of Pitt and Hocking (40) and genetic analysis (see below). Once the strains were identified they were named as follows: *P. nalgiovense* 16a, *P. commune* INBCC300 (25), and *P. chrysogenum* AK (all isolated from cecina); and *P. roqueforti* CONT1 (isolated from blue cheese).

P. chrysogenum AS-P-78 was kindly donated by Antibióticos, S.A. (León, Spain) (11). *P. nalgiovense* INBCC103 is a commercial starter culture.

Identification procedures. All isolates were identified to the species level according to the method of Pitt and Hocking (40). To confirm this identification, the randomly amplified polymorphic DNA (RAPD) technique was used. Total DNA was obtained as described by Fierro et al. (12). RAPD PCR amplification was carried out according to the protocol described by Williams et al. (45) with modifications. PCRs were performed with a 25-μl volume containing the following: 16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 0.01% Tween 20; 3.5 mM $MgCl_2$; a 200 μM concentration (each) of dATP, dCTP, dGTP, and dTTP (Fermentas); 0.32 μM primer; 0.5 U of *Taq* DNA polymerase (Bioline); and 10 to 20 ng of genomic DNA. The following oligonucleotides were used: 1, 5′-AGTCAGCCAC-3′; 2, 5′-AATCGGGCT-3′ (24); CRL9, 5′-CAGCCGCCCC-3′; and CRL12, 5′-CCGCCGCCCG-3′ (5). PCR was carried out with a Whatman Biometra T Gradient thermal cycler using an initial denaturation step of 94°C for 4 min; followed by 45 cycles of 40 s at 94°C, 1 min at 34°C, and 2 min at 72°C; and a final extension of 72°C for 10 min. Amplified fragments were separated on a 1.5% (wt/vol) agarose gel and visualized by ethidium bromide staining.

Bioassays for penicillin production. All the strains described above were tested for their ability to produce penicillin by bioassays in both solid and liquid medium with *Micrococcus luteus* ATCC 9341 as a test strain. β-Lactamase from *Bacillus cereus* UL1 (300 μl from the supernatant of a *B. cereus* culture per 100 ml of tryptic soy agar [TSA] medium) was used to check if the antibacterial activity observed in the samples was due to the presence of penicillin or to some other antagonistic compound. The bioassay on solid medium was performed by growing the fungus in petri dishes on MEA at 25°C for 7 to 10 days (when a high number of conidia was produced by the colony). Agar plugs (9-mm diameter) were taken out of the fungal colonies, placed on the surface of TSA medium (Difco) containing 1% agar, and incubated for 48 h at 25°C. Then, an overlayer of 1% TSA with *M. luteus* was added onto the medium with the plugs, the antibiotic was allowed to diffuse in the plates in the cold (2 h at 5°C), and finally the cultures were incubated at 30°C for 24 h.

Studies of the production of penicillin on liquid cultures were carried out as described previously (25).

HPLC analysis. *P. griseofulvum* NRRL 2300 was fermented on a liquid culture in CPM medium under the conditions described before (25). Samples from the supernatant were taken after 96 h of fermentation. Fifty microliters of both the unprocessed supernatant and an ethyl acetate-extracted fraction obtained as described previously by Laich et al. (25) was analyzed with a Waters high-performance liquid chromatograph (HPLC) with a μBondapak C_{18} column. Penicillin G at a concentration of 0.1 mg/ml was used as the control sample. Buffer A was 50 mM sodium acetate, pH 4.5, and buffer B was acetonitrile. The flow rate was 1.3 ml/min, and the running conditions were as follows: min 1 to 3, buffer A; min 3 to 15, gradient buffer B, 0 to 60%; min 15 to 18, gradient buffer B, 60 to 80%; min 18 to 20, buffer B, 80%; min 20 to 22, gradient buffer B, 80 to 0%; and min 22 to 25, buffer A.

Southern blotting and hybridization. Total DNA of the fungi was extracted as described before (12). Five micrograms of DNA of each strain was digested with the restriction endonuclease *Eco*RI. The DNA fragments were separated on a horizontal 0.6% agarose gel and transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech) using a vacuum blotter (VacuGene XL; Pharmacia Biotech). The probes (see Fig. 5) were labeled with the DIG DNA labeling mixture (Boehringer Mannheim) according to the manufacturer's protocol. Prehybridization and hybridization were done with 40% formamide standard buffer (41) at 42°C. After hybridization, the membrane was washed at room temperature for 15 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS), 15 min at 42°C with 0.1× SSC–0.1% SDS, and 3 min at 65°C with 0.1× SSC–0.1% SDS. The signals were visualized with a chemiluminescent substrate for alkaline phosphatase (Roche) according to the manufacturer's protocol.

PCR amplification and sequencing. The following primers were used for amplification by PCR: NALAB1, 5′-CTTCTCGAGGGGCTAGTCATGAGTCCGTATTCA-3′; NALAB2, 5′-CAAGGATCCTTGGAATCACACTTTCTACGTCCG-3′; PVCG, 5′-RTCRATDCKDCCSAGRTACTCRAT-3′; PVCB, 5′-GGHGGHGCYTAYGTBCCNATYGA-3′; PCAB1, 5′-CTGGATATCCTAACGACCGC-3′; and PCAB2, 5′-CAGGTAGAGCTCGCCGACAG-3′.

The letter code used for nucleotide designation is according to the Nomenclature Committee of the International Union of Biochemistry (37).

PCR amplification was performed by standard procedures. In the case of primers PVCG and PVCB, an annealing temperature gradient between 46 and 69°C was used.

DNA sequencing was performed with an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) using standard sequencing primers and the ABI PRISM sequencing kit, according to the manufacturer's instructions.

TABLE 1. Antibacterial activity of fungal strains grown on MEA solid medium in this study[a]

Strain	Diam of the inhibition zone (mm)[b]	
	Without β-lactamase	With β-lactamase
P. chrysogenum AK	49 ± 1.5	0
P. nalgiovense 16a	38.3 ± 1.4	0
P. griseofulvum NRRL 2300	47 ± 1.4	0
P. griseofulvum NRRL 994	22.8 ± 1.3	0
P. griseofulvum NRRL 2152	22.2 ± 1.1	0
P. griseofulvum NRRL 992	32.6 ± 1.1	29.4 ± 0.9
P. griseofulvum NRRL 989	27.2 ± 1.0	24.2 ± 1.2
P. griseofulvum NRRL 991	33.6 ± 1.3	32.8 ± 1.2
P. verrucosum NRRL 5574	13 ± 0.8	13 ± 0.9
P. verrucosum NRRL 5573	15 ± 0.6	15 ± 0.5
P. crustosum NRRL 972	16.6 ± 1.1	21 ± 1.0

[a] Only the strains with some antibacterial activity are listed.
[b] Values are the average of five different experiments ± standard deviation.

RESULTS

Penicillin production by different strains. All the strains were first screened to detect penicillin producers using a very sensitive bioassay in solid medium. No antibacterial activity was observed after five bioassays of strains belonging to the species *P. roqueforti*, *P. camemberti*, *P. brevicompactum*, *P. commune*, *P. solitum*, *P. expansum*, *P. implicatum*, *P. hirsutum*, *P. aurantiogriseum*, *P. viridicatum*, *P. echinulatum*, *P. purpurogenum*, and *Paecilomyces variotii*. However, strains belonging to *P. chrysogenum*, *P. nalgiovense*, *P. griseofulvum*, *P. crustosum*, and *P. verrucosum* consistently showed different levels of antibacterial activity (Table 1). The highest antibacterial activity was found in *P. chrysogenum* AK, followed by *P. griseofulvum* NRRL 2300 and by *P. nalgiovense* 16a. β-Lactamase from *B. cereus* UL1 was used as a means to discern between the antibacterial activity caused by penicillin and that exerted by other antagonistic substances that the fungus might produce. The results shown in Table 1 indicate that in *P. chrysogenum*, *P. nalgiovense*, and three strains of *P. griseofulvum*, all the antibacterial activity can be attributed to a β-lactam antibiotic, presumably penicillin. However, in three other strains of *P. griseofulvum* (NRRL 991, NRRL 992, and NRRL 989) only part of the antibacterial activity could be attributed to penicillin, as a smaller penicillinase-resistant inhibition halo was still formed in the presence of β-lactamase (see Discussion). In *P. verrucosum* and *P. crustosum*, no decrease of the inhibition halo was observed upon treatment with β-lactamase, which indicates that the antibacterial activity observed in this case is not caused by penicillin but by some other compound(s).

Penicillin production by *P. griseofulvum* strains in solid medium was confirmed by fermentation in liquid submerged cultures (Fig. 1). *P. griseofulvum* strain NRRL 2300 showed the

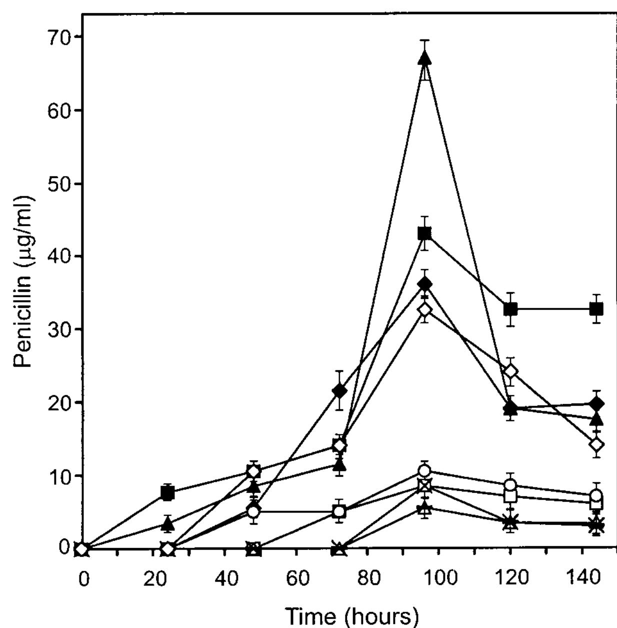

FIG. 1. Penicillin production in liquid submerged cultures of the strains shown to produce an antibacterial compound that was inactivated by β-lactamase (see Table 1 and text). Strains depicted here are *P. nalgiovense* 16a (◆), *P. nalgiovense* INBCC103 (commercial starter culture) (■), *P. chrysogenum* AK (▲), *P. griseofulvum* NRRL 991 (□), *P. griseofulvum* NRRL 2152 (–), *P. griseofulvum* NRRL 994 (○), *P. griseofulvum* NRRL 992 (×), *P. griseofulvum* NRRL 989 (□), and *P. griseofulvum* NRRL 2300 (◇). Error bars, standard deviations.

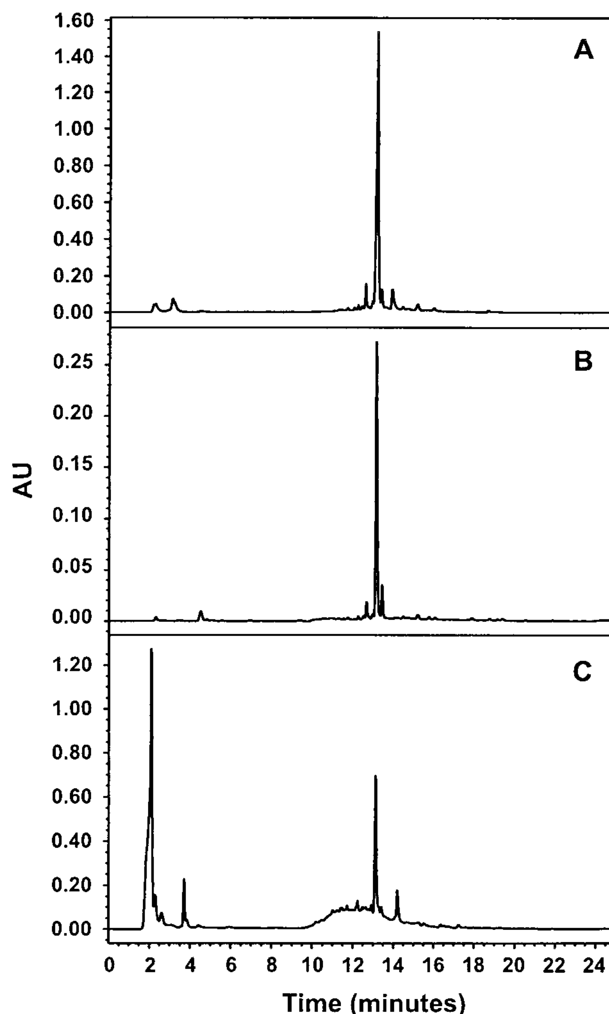

FIG. 2. HPLC analysis of the supernatant of a fermentation of *P. griseofulvum* NRRL 2300. (A) Control sample of penicillin G at a concentration of 0.1 mg/ml. The retention time for the penicillin G peak was 13.18 min. (B) Chromatogram of an ethyl acetate-extracted sample of that supernatant. (C) Chromatogram of the unprocessed supernatant. The extraction with ethyl acetate partially purifies and enriches the penicillin G in the sample.

highest production level, while the rest of the *P. griseofulvum* strains produced much smaller amounts (less than 10 μg/ml). In some of the strains, penicillin production was detected only after 80 h of fermentation.

An HPLC analysis of the supernatant of a *P. griseofulvum* NRRL 2300 liquid culture was performed to confirm that the antibiotic produced by this fungus was penicillin. As shown in Fig. 2, a peak with a retention time of 13.2 min that corresponds to the control sample of penicillin G was observed in both the unprocessed supernatant and in the ethyl acetate extract. It was, therefore, concluded that *P. griseofulvum* produces authentic penicillin G.

Analysis of the presence of penicillin biosynthetic genes in different strains. The results on production of penicillin observed in the previous experiments should correlate with the presence or absence of the penicillin biosynthetic genes *pcbAB*, *pcbC*, and *penDE* in the different strains. We performed a series of Southern blot analyses to detect the possible presence of the genes in all the strains used in this study. The probes used for these experiments are shown in Fig. 5. Probes C, D, and E correspond to the *pcbAB* gene, encoding L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) synthetase, the multidomain enzyme catalyzing the first step of the biosynthesis of penicillin. Probes A and B correspond to the *pcbC* and *penDE* genes, respectively, which encode the enzymes responsible for the second and third step of the biosynthesis. The results obtained with probes A and B (Fig. 3) indicate that both the *pcbC* and the *penDE* genes are present in all the strains of *P. chrysogenum*, *P. nalgiovense*, and *P. griseofulvum* and are absent in all the remaining strains belonging to other species. The presence of the penicillin gene cluster confirms that *P. griseofulvum* is a penicillin producer. Similarly, the absence of the penicillin cluster in *P. verrucosum* and *P. crustosum* supports the conclusion that the antibacterial activity observed in these fungi is not due to penicillin. All the strains of *P. griseofulvum* contained the whole *pcbAB* gene, as revealed by the hybridization signals appearing with the three probes C, D, and E (Fig. 4). The Southern blot analyses performed with these five probes, along with additional Southern blots with other probes from the cluster, allowed us to elaborate on an *Eco*RI restriction map of the new penicillin gene cluster in *P. griseofulvum* by comparison with the previously described maps of *P. chrysogenum* (6) and *P. nalgiovense* (25). As shown in Fig. 5, there is an *Eco*RI site in the *penDE* gene of the *P. griseofulvum* NRRL 991 strain which is absent in the other strains of *P. griseofulvum*. In gen-

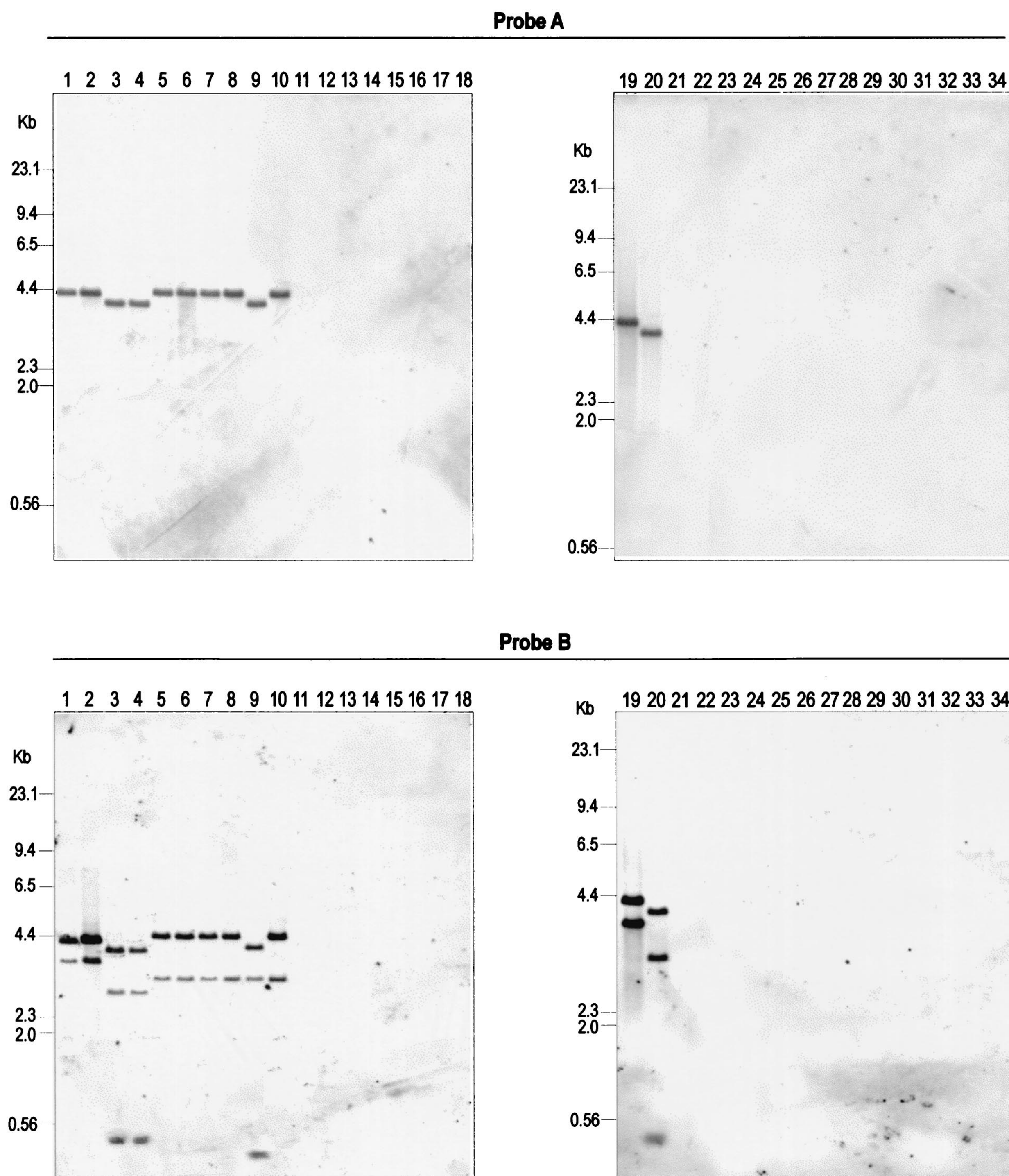

FIG. 3. Presence of the genes *pcbC* and *penDE* in penicillin-producing strains and absence in nonproducing strains. *Eco*RI-digested genomic DNA from all the strains under study was electrophoresed (lanes 1 to 34), transferred to a nylon membrane, and hybridized with probe A (corresponding to the *pcbC* gene) and probe B (corresponding to the *penDE* gene). Lanes: 1 and 2, *P. chrysogenum* NRRL 1951 and AS-P-78; 3 and 4, *P. nalgiovense* NRRL 911 and 16a; 5 to 10, *P. griseofulvum* NRRL 2300, NRRL 2152, NRRL 989, NRRL 994, NRRL 991, and NRRL 992; 11 and 12, *P. roqueforti* CONT1 (isolated from blue cheese) and NRRL 849; 13, *Paecilomyces variotii* NRRL 1775; 14 to 17, *P. camemberti* NRRL 874, NRRL 877, NRRL 876 (ex-type of *P. candidum*), and CECT 2267; 18, *P. commune* INBCC300 (isolated from cecina); 19, *P. chrysogenum* AS-P-78; 20, *P. nalgiovense* 16a; 21 and 22, *P. verrucosum* NRRL 5573 and NRRL 5574; 23, *P. brevicompactum* NRRL 862; 24 and 25, *P. commune* NRRL 845 and CEC1 (isolated from cecina); 26, *P. solitum* NRRL 937; 27, *P. crustosum* NRRL 972; 28, *P. expansum* NRRL 976; 29, *P. implicatum* NRRL 2061; 30, *P. hirsutum* NRRL 2032; 31, *P. aurantiogriseum* NRRL 971; 32, *P. viridicatum* NRRL 963; 33, *P. echinulatum* NRRL 1151; 34, *P. purpurogenum* ATCC MYA-38.

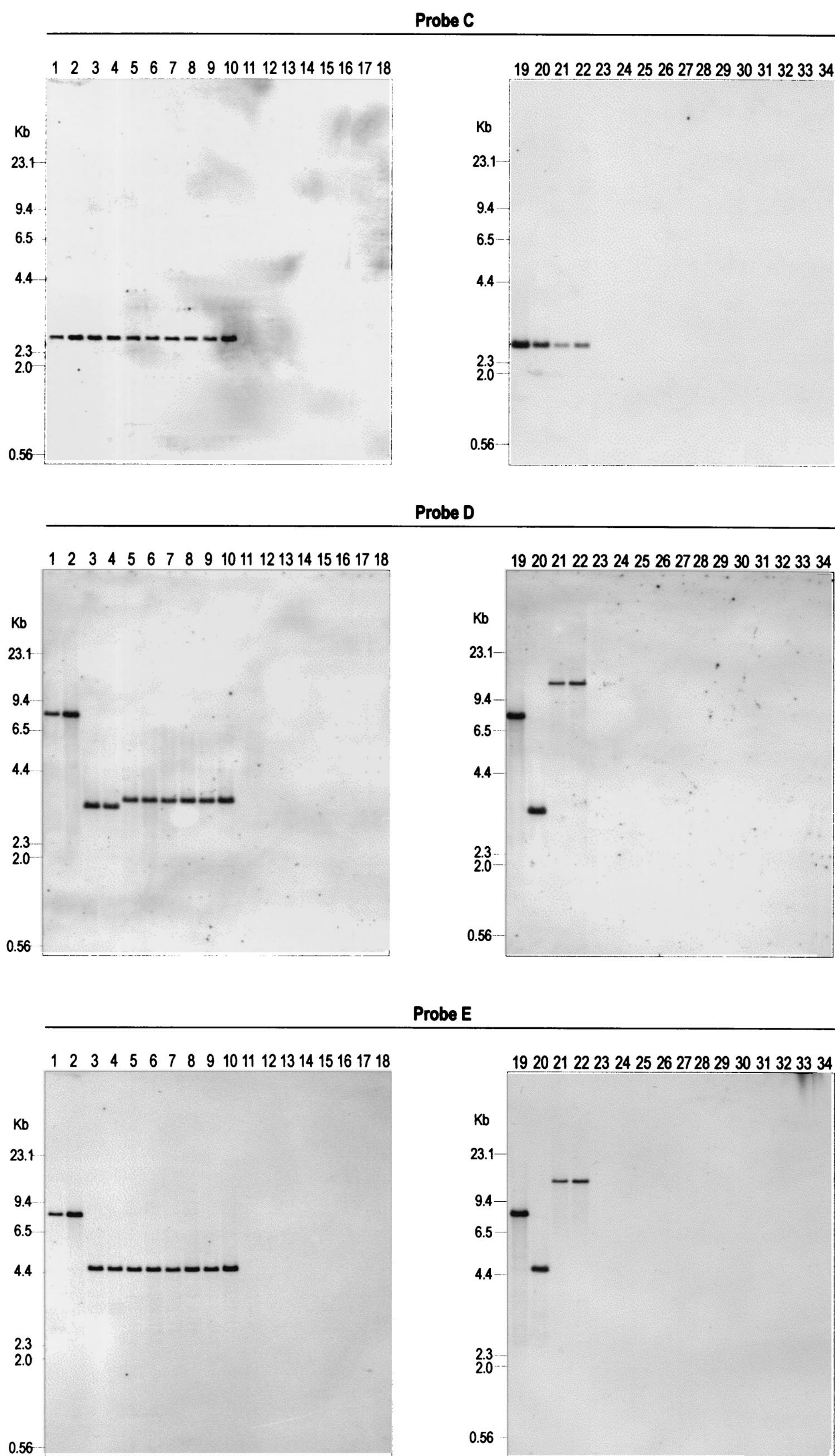

FIG. 4. Presence of the gene *pcbAB* in penicillin-producing strains. *Eco*RI-digested genomic DNA from all the strains was electrophoresed (lanes 1 to 34); transferred to a nylon membrane; and hybridized with probes C, D, and E, which correspond to different portions of the *pcbAB* gene (Fig. 5). Strain numbers are as indicated in the legend to Fig. 3. Note that in *P. verrucosum* (lanes 21 and 22) there is a signal of 2.5 kb with probe C, which reveals the presence of the 3′ end of the *pcbAB* gene in this species. With probes D and E, a signal of about 12.5 kb can be observed that corresponds also to the *pcbAB* gene (see text).

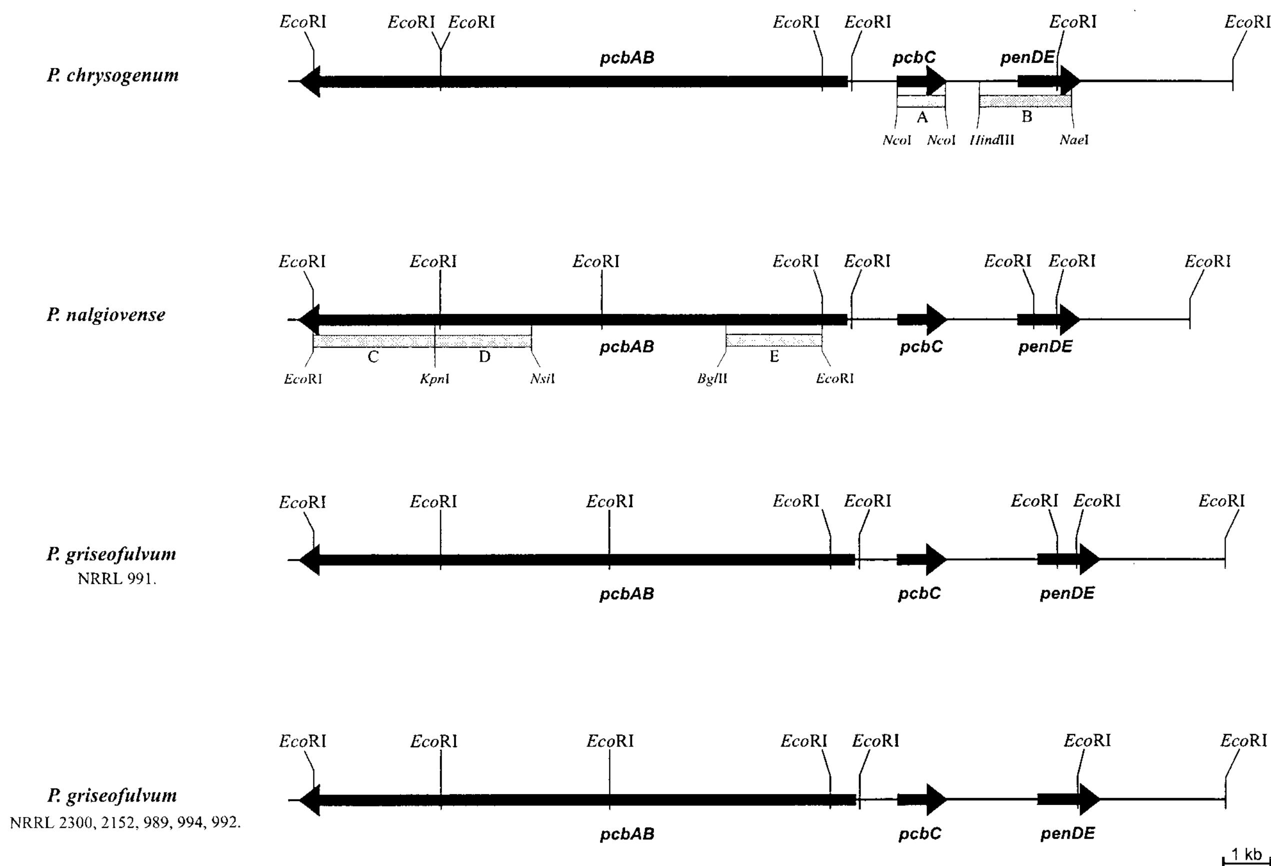

FIG. 5. *Eco*RI restriction map of the penicillin gene cluster in *P. chrysogenum*, *P. nalgiovense*, and six different strains of *P. griseofulvum*. The five probes used in the Southern analysis are indicated as grey boxes below the maps of *P. chrysogenum* and *P. nalgiovense*, together with the enzymes used to obtain the probes. Most *Eco*RI sites are conserved between the three species, except the site at the 5′ end of the *penDE* gene, which is present only in *P. nalgiovense* and *P. griseofulvum* NRRL 991, but in a slightly different position in each of these two strains. An *Eco*RI fragment inside the *pcbAB* gene (overlapping partially with probe D) shows a slightly larger size in *P. griseofulvum* than in *P. nalgiovense*. A similar phenomenon occurs in the right part of the cluster, where the *Eco*RI fragment comprising the *pcbC* gene and most of the *penDE* gene is slightly larger in *P. griseofulvum* than in *P. chrysogenum*.

eral most of the restriction sites are conserved between the three species, but it is striking that one of the *Eco*RI fragments inside *pcbAB* is about 160 bp larger in *P. griseofulvum* than in *P. chrysogenum* (Fig. 4 and 5).

The existence of differences in the restriction map of *P. griseofulvum* NRRL 991 with respect to the rest of the *P. griseofulvum* strains prompted us to confirm by RAPD experiments that all of the *P. griseofulvum* collection strains had been correctly identified (Fig. 6). The pattern of bands obtained with the primers used (see Materials and Methods) clearly indicated that all the strains belonged to the species *P. griseofulvum*; therefore, the change in the restriction map of strain NRRL 991 is just a consequence of the genetic variability between *P. griseofulvum* strains.

In *P. verrucosum* a striking result was obtained with probes C, D, and E. A 2.5-kb hybridization signal apparently belonging to the *pcbAB* gene was observed with probe C, which corresponds to the 3′ end of *pcbAB*, whereas with probes D (adjacent to probe C) and E (corresponding to the 5′ end of the gene) a signal of about 12.5 kb was found that did not coincide with the *Eco*RI restriction map of the *pcbAB* gene of the other species (Fig. 4, lanes 21 and 22). The 12.5-kb signal could be explained either by the presence of the *pcbAB* gene with a different *Eco*RI restriction map (where only the two *Eco*RI sites at the 3′ end of the gene had been conserved) or by a phenomenon of heterologous hybridization with another gene having a high degree of similarity with the *pcbAB* gene. To further investigate these possibilities, different DNA fragments from *P. verrucosum* genomic DNA were PCR amplified and sequenced.

Analysis of the *P. verrucosum pcbAB* gene. First, we studied whether the 2.5-kb *Eco*RI fragment located at the 3′ end of the *pcbAB* gene was present in *P. verrucosum*, as suggested by the result of the hybridization with probe C (Fig. 4). For this purpose the primers NALAB1 and NALAB2, designed according to the sequence of the *P. chrysogenum pcbAB* gene (situated at bp 9410 and 10430, respectively, from the ATG) (6), were used to amplify by PCR a DNA fragment of approximately 1 kb from *P. verrucosum*, included within the 2.5-kb *Eco*RI fragment. Partial sequence analysis of the amplified fragment showed 95% identity with the *P. chrysogenum* gene at the DNA level, thus confirming the presence of the 3′ end of the *pcbAB* gene in *P. verrucosum*.

Another pair of primers, PCAB1 and PCAB2, designed also

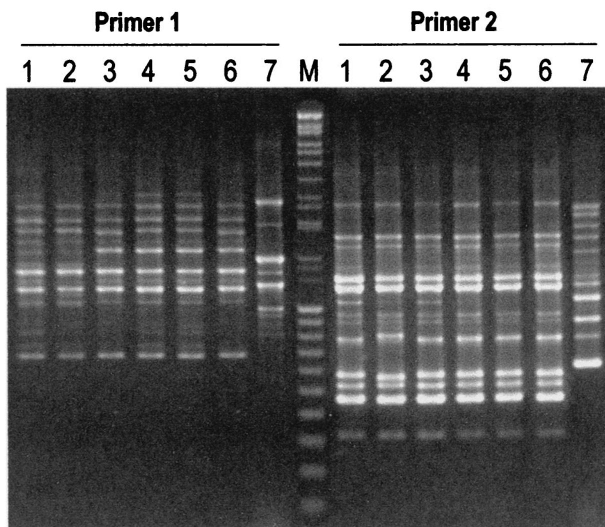

FIG. 6. RAPD analysis of seven strains previously classified as *P. griseofulvum*. Primers and PCR conditions are indicated in Materials and Methods. The pattern of bands shown by the different strains clearly indicated that all of them belonged to the species *P. griseofulvum*. Lanes: 1, *P. griseofulvum* NRRL 2300; 2, NRRL 991; 3, NRRL 992; 4, NRRL 994; 5, NRRL 989; 6, NRRL 2152; 7, *P. nalgiovense* NRRL 911; M, size marker.

from the *P. chrysogenum pcbAB* sequence and belonging to the central part of the gene (located at bp 4497 and 5291, respectively, from the ATG) between probes D and E was used to test the presence of that portion of the *pcbAB* gene in *P. verrucosum*. Genomic DNAs from *P. chrysogenum* NRRL 1951, *P. nalgiovense* NRRL 911, and *P. griseofulvum* NRRL 2300 and NRRL 991 were used as control templates in the PCR. A fragment of the expected size, about 0.8 kb, was amplified with different intensities in all the strains, though in *P. verrucosum* additional fragments were also amplified (not shown). The *P. verrucosum* 0.8-kb fragment was subcloned and partially sequenced, showing also a very high percentage of identity (96%) at the DNA level to the corresponding fragment of the *P. chrysogenum pcbAB* gene. This result confirmed the presence of the central part of the *pcbAB* gene (corresponding to the second activating domain of the enzyme) in *P. verrucosum*.

The *pcbAB* gene encodes ACV synthetase, an enzyme belonging to the family of the nonribosomal peptide synthetases. These enzymes are organized in domains, each activating one amino acid. In each domain there are several conserved boxes that have different functions in the enzyme (21, 35). To test whether the hybridization signal of 12.5 kb observed in *P. verrucosum* with probes D and E was due to a gene with a high degree of identity with the *pcbAB*, the following approach was developed. Two degenerated oligonucleotides, PVCB and PVCG, were designed according to the amino acid sequence of boxes B and G, following the codon usage of *P. chrysogenum*. With these primers, a temperature gradient PCR was performed. Several bands amplified; among these, one of the expected size for the *pcbAB* gene domains (1.0 kb) became predominant at higher temperatures. This band when subcloned was shown to be composed of two different DNA fragments, which corresponded, respectively, to positions 1190 to 2197 and 4472 to 5467, numbered from the *P. chrysogenum pcbAB* ATG start codon. The sequence of these fragments revealed that they corresponded, respectively, to the first and second activating domains of the ACV synthetase, and showed a high degree (92 to 96%) of identity at the DNA level with the *pcbAB* gene of *P. chrysogenum*. Eleven other amplified fragments of different sizes were subcloned and sequenced. Their sequences did not show significant identity with peptide synthetases. Therefore, we concluded that the 12.5-kb hybridization signal in *P. verrucosum* corresponds to the *pcbAB* gene and that this gene is present apparently in all its length in this fungus; no evidence for the possible presence of additional peptide synthetase genes in *P. verrucosum* was obtained.

DISCUSSION

Among different fungal species belonging to the genus *Penicillium* that are currently used as starter cultures in the food industry or that are frequently isolated from cured meat products, only *P. griseofulvum* (in addition to the previously reported *P. chrysogenum* and *P. nalgiovense*) was found to produce penicillin and possess the three penicillin biosynthetic genes (*pcbAB*, *pcbC*, and *penDE*). It is important that the commonly used cheese starters *P. camemberti* and *P. roqueforti* neither are penicillin producers nor possess the penicillin biosynthetic genes, which implies that they do not represent a risk regarding the problem of the presence of penicillin in food.

P. griseofulvum is frequently isolated from corn, wheat, barley, flour, and walnuts (40) and from meat products (27), thus being a potential source for the presence of penicillin in food. The antibacterial activity detected in six *P. griseofulvum* strains used in the present study was sensitive to β-lactamase, indicating that *P. griseofulvum* is a penicillin producer, which was confirmed by HPLC (Fig. 2). However, in three of these *P. griseofulvum* strains—NRRL 991, NRRL 992, and NRRL 989—an antibacterial activity could still be detected in the presence of excess β-lactamase, suggesting that another compound with antibacterial activity must be produced and secreted to the medium in these strains. *P. griseofulvum* has been described as synthesizing four mycotoxins: patulin, cyclopiazonic acid, roquefortine C, and griseofulvin (8, 15). The polyketide-derived mycotoxin patulin possesses a strong antibacterial activity that might well account for the β-lactamase-resistant antibacterial activity found in the aforementioned strains; cyclopiazonic acid and roquefortine C have also been described as antibacterial substances (22, 32) and could contribute to that antibacterial activity.

P. griseofulvum must, therefore, be added to the list of *Penicillium* species with the ability to produce penicillin, along with *P. chrysogenum*, *P. nalgiovense*, *Penicillium dipodomys*, and *Penicillium flavigenum* (3, 7, 10, 14, 15, 16, 25, 43). These four *Penicillium* species are very closely related and belong to the *P. chrysogenum* complex of taxonomically related xerophilic penicillia. Banke et al. (4) classified them as separated species on the basis of isozyme analysis.

P. verrucosum represents a special case among the studied fungi. In contrast to other strains that do not produce penicillin, *P. verrucosum* strains contain one of the genes (*pcbAB*) of the penicillin biosynthesis gene cluster. This is most likely reminiscent of the former capacity of this fungus to produce

penicillin. The origin of the penicillin gene cluster in fungi has been proposed to have occurred by a horizontal gene transfer of the genes *pcbAB* and *pcbC* from β-lactam-producing bacteria (2, 26, 30, 38, 44). Since the structure and orientation of the genes in the penicillin gene cluster is the same in both *Aspergillus* (33) and *Penicillium* (6, 25) species the most likely hypothesis is that the cluster was formed before the split between *Aspergillus* and *Penicillium* occurred (13), therefore implying that all the species belonging to the genus *Penicillium* that lack the ability to produce penicillin have lost this ability throughout the evolution from a common ancestor able to synthesize the antibiotic. The penicillin gene cluster may have been lost in different moments in the distinct *Penicillium* lineages, either the three genes at the same time or just part of the cluster, as seems to have happened in *P. verrucosum*.

The antibacterial activity observed with *P. verrucosum* may be due to the production of patulin or penicillic acid, as both secondary metabolites have been reported to be produced by strains of this fungus (47).

There is a high degree of conservation of the *Eco*RI restriction sites in the penicillin gene cluster of the different strains studied. In this regard, the *P. griseofulvum* restriction map shares resemblance with both *P. chrysogenum* and *P. nalgiovense* maps (Fig. 5). This feature is due to the high degree of identity between the penicillin genes in the different *Penicillium* species. The transcription start point of the *pcbAB* is situated adjacent to the *Eco*RI site in the *P. chrysogenum* promoter (23), so this DNA region is probably functionally important in all strains. In *P. verrucosum*, only the two *Eco*RI sites at the 3′ end of the *pcbAB* gene have been conserved, while the rest of the sites are no longer present. The absence of the *Eco*RI sites in the 5′ part of the *P. verrucosum* gene might be attributed to mutations that have accumulated after the penicillin gene cluster stopped being functional.

Studies of antibiotic and toxin production by food microorganisms are important for obtaining safer and higher-quality food products. The knowledge of their biosynthetic pathways and genes will allow us to obtain modified strains to be used as starters, which will conserve their food-ripening and flavoring properties while preventing the synthesis of toxic compounds that might eventually be secreted to the food product.

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