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Antagonistic Activity of the Food-Related Filamentous Fungus *Penicillium nalgiovense* by the Production of Penicillin

P. FÄRBER AND R. GEISEN*

Federal Research Centre for Nutrition, Institute for Hygiene and Toxicology, 76131 Karlsruhe, Germany

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Defined strains of the genus *Penicillium* used as starter cultures for food and strains isolated from mold-fermented foods were analyzed for their ability to inhibit the growth of *Micrococcus luteus* DSM 348 used as an indicator organism. Most of the strains belonging to the species *Penicillium nalgiovense* showed antagonistic activity in agar diffusion assays. *Penicillium camemberti* and *Penicillium roqueforti* strains proved to be inactive in these tests. The inhibitory substance excreted by *P. nalgiovense* strains was totally inactivated when treated with β-lactamase (penicillinase), indicating that a β-lactam antibiotic is produced by these strains. This observation was verified by PCRs with primer sets specific to the [δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine] synthetase gene (*pcbAB*), the isopenicillin-*N*-synthase gene (*pcbC*), and the acyl coenzyme A:6-aminopenicillanic acid acyltransferase gene (*penDE*) from *Penicillium chrysogenum* using chromosomal DNA of the fungal strains as a template. These results indicate that penicillin biosynthesis is a characteristic often found in strains of *P. nalgiovense*. No specific PCR signal could be identified with DNA from *P. camemberti* and *P. roqueforti*.

Different species of the genus *Penicillium* are used in the European food industry for the production of mold-fermented foods. Particularly, *P. camemberti* is used for white cheeses, *P. roqueforti* is used for blue-veined cheeses, and *P. nalgiovense* is used for meat products (4). Fungal strains must fulfill certain requirements before being used as starter cultures. Most importantly, they should be nontoxinogenic and should not produce any antibiotics. Strains of the genus *Penicillium* are often able to produce mycotoxins, a chemically diverse group of secondary metabolites. *P. camemberti*, for example, is able to produce cyclopiazonic acid, whereas strains of *P. roqueforti* are well-known producers of patulin, pathogenesis-related toxin, mycophenolic acid, and roquefortin C (7). In contrast to these two species, *P. nalgiovense* has, to our knowledge, never been reported to produce mycotoxins. Antibiotics, like the β-lactam antibiotic penicillin, are another group of secondary metabolites. Differences between these two groups are only qualitative. The genes coding for penicillin biosynthetic enzymes have been elucidated. Three genes are responsible for the biosynthesis of penicillin (1). In particular, *pcbAB* codes for [δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine] synthetase, *pcbC* codes for isopenicillin-*N*-synthase, and *penDE* encodes acyl coenzyme A:isopenicillin-*N*-acyltransferase complex. The nucleotide sequences of all three genes are known (2, 3, 6).

In this publication, the ability of *P. nalgiovense* to produce penicillin is reported and evidence for the existence of the genes responsible for penicillin production is given.

MATERIALS AND METHODS

Strains and culture conditions. A list of some typical fungal strains analyzed in this study is given in Table 1. Throughout this study, *Micrococcus luteus* DSM 348 was used as a bacterial indicator organism. Fungal strains were routinely grown on malt extract medium (E. Merck, Darmstadt, Germany). For determination of inhibitory activity, indicator bacteria were cultivated on standard I medium (E. Merck). Strains of *P. nalgiovense* were incubated at 25°C; *M. luteus* DSM 348 was incubated at 37°C.

Determination of inhibitory activity. The inhibitory activity was determined by two different methods. Agar plug tests were performed for a rapid qualitative analysis of the ability of fungal strains to produce antagonistic substances. Agar plugs from 5- to 7-day-old fungal colonies were transferred to plates, inoculated with cells from an overnight culture of *M. luteus* DSM 348, and incubated overnight at 37°C. Strains showing antagonistic activity were transferred from the original plates to liquid minimal medium (medium composition per liter: glucose, 5.0 g; KH_2PO_4, 3.75 g; $MgSO_4$, 0.5 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; KOH, 0.75 g; KNO_3, 1.2 g) and incubated under aeration for 5 days. After that time the culture was filtered through Miracloth. For semiquantitative determination of penicillin activity, a volume of 300 μl of the cell-free supernatants of these cultures was transferred to standard I agar plates prepared with a well and overnight diffusion was allowed. A second sample of the supernatant was mixed with β-lactamase (Sigma, St. Louis, Mo.) and applied in the same way. These agar plates were inoculated with 100 μl of an overnight culture (dilution, $1:10^3$) of the indicator bacterium *M. luteus* DSM 348 and incubated overnight at 37°C. Additionally, a part of the mycelium was applied to a plate and inoculated with the indicator organism in the same way (Fig. 1).

Isolation of fungal DNA. The isolation of DNA from fungal strains was performed according to a modified method originally described by Yelton et al. (11). For that purpose, 72- to 96-h-old mycelium was harvested from a submersed culture by filtration. The mycelium was transferred to a mortar and frozen in liquid nitrogen. The frozen mycelium was ground and then resuspended in lysis buffer (50 mM EDTA, 0.2% sodium dodecyl sulfate, pH 8.5). This suspension was heated to 68°C for 15 min and then centrifuged for 15 min at 15,000 × *g*. After centrifugation a volume of 7 ml of the supernatant was transferred to a new centrifuge tube, and 1 ml of 4 M sodium acetate was added. This solution was placed on ice for 1 h and

* Corresponding author. Mailing address: Federal Research Centre for Nutrition, Institute for Hygiene and Toxicology, Engesserstr. 20, 76131 Karlsruhe, Germany. Phone: 49 7247 823619.

TABLE 1. Sources, inhibitory activities, and occurrences of *pcbAB*, *pcbC*, and *penDE* gene-specific PCR products of exemplary selected fungal strains

BFE strain	Species	Source[a]	Presence of[b]:			
			Inhibitory activity	ACV	IPNS	AAT
236	*P. chrysogenum*	DSM 1075	+	+	+	+
66	*P. nalgiovense*	ATCC 66742	+	+	+	+
71	*P. nalgiovense*	BFE culture collection	+	+	+	+
61	*P. nalgiovense*	BFE culture collection	−	−	(+)[c]	−
57	*P. nalgiovense*	BFE culture collection	+	+	+	+
233	*P. nalgiovense*	Commercial starter culture	+	+	+	+
247	*P. nalgiovense*	Isolated from salami (France)	+	+	+	+
249	*P. nalgiovense*	Isolated from soft cheese (Germany)	+	+	+	+
255	*P. nalgiovense*	Isolated from salami (Italy)	+	+	+	+
224	*P. camemberti*	Commercial starter culture	−	−	−	−
63	*P. roqueforti*	BFE culture collection	−	−	−	−

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures); ATCC, American Type Culture Collection; BFE, Bundesforschungsanstalt für Ernährung (Federal Research Centre for Nutrition).

[b] ACV, PCR fragment (550 bp) of [δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine] synthetase gene; IPNS, PCR fragment (600 bp) of isopenicillin-*N*-synthase gene; AAT, PCR fragment (650 bp) of acyl coenzyme A:6-aminopenicillanic acid acyltransferase gene; +, present; −, absent.

[c] PCR fragment length larger than expected (>600 bp).

centrifuged for 15 min at 15,000 × *g*. After centrifugation, 6 ml of the supernatant was transferred to a fresh tube and the isolated DNA was precipitated.

PCR. The isolated chromosomal DNA was diluted to 2 μg/ml and used as the template DNA for PCRs specific for penicillin biosynthetic genes. A typical PCR mixture contained 5.0 μl of *Taq* polymerase buffer (10×), 4.0 μl of $MgCl_2$ (25 mM), 1.25 μl of primer (120 pmol/μl, each primer), 1.0 μl of *Taq* polymerase (5 U/μl; Promega, Madison, Wis.), 5.0 μl of template DNA, and 25.0 μl of H_2O. Primer sets of the following sequences were synthesized: acv1, 5′TCGTGCTG GATGACACCAAGGCACG3′; acv2, 5′CACCAGGATTAT CCGATTCAGTGAT3′, enclosing a fragment of 550 bp of the *P. chrysogenum pcbAB* gene from nucleotides 1501 to 2050 (6); ipns1, 5′TGTGGCCGGACGAGAAGAAGCATCC3′; ipns2, 5′TCTTGTTGATTAGACTAACTAATCC3′, enclosing a fragment of 600 bp of the *P. chrysogenum pcbC* gene from nucleotides 801 to 1400 (2); aat1, 5′ACCAAAGAGAACCT GATCCGGTTAA3′; and aat2, 5′ATGACAAACATCTCAT CAGGGTTGG3′, enclosing a fragment of 650 bp of the *P. chrysogenum penDE* gene from nucleotides 1051 to 1700 (3). PCRs were performed in 35 cycles of 1 min at 95°C, 3 min at 65°C, and 4 min at 72°C (Thermocycler Gene ATAQ Controller; Pharmacia, Uppsala, Sweden).

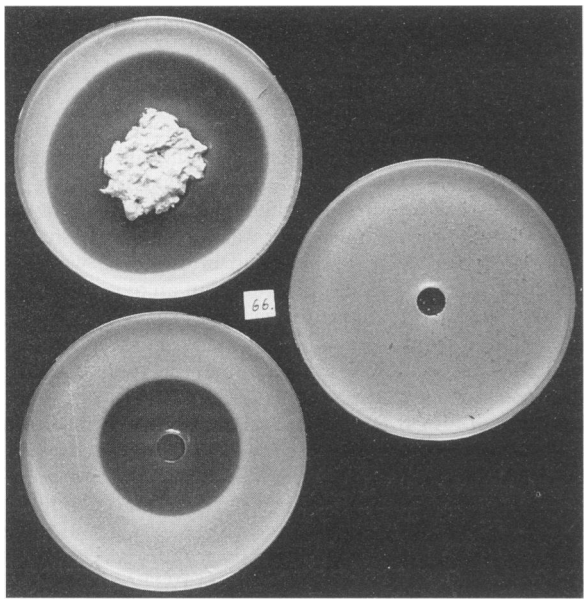

FIG. 1. Agar diffusion assay demonstrating growth inhibition of the indicator bacterium *M. luteus* DSM 348 caused by *P. nalgiovense* BFE 66. (Left) Inhibitory activity of mycelium (upper plate) and culture supernatant (lower plate); (right) inactivation of the antagonistic substance after β-lactamase treatment.

Ultrafiltration. Ultrafiltration experiments were carried out at 4°C using a series 8400 stirred ultrafiltration cell (Amicon, Beverly, Mass.). The mycelium of a 96- to 120-h-old culture was first removed by a filtration step, and 200 ml of the supernatant was used for the ultrafiltration processes. For the enrichment of the inhibitory substance, different membranes have been used: Amicon membranes YM10 (molecular weight cutoff, 10,000), YM5 (molecular weight cutoff, 5,000), and YM2 (molecular weight cutoff, 1,000). Maximum nitrogen pressure was 3.8×10^5 Pa. After each ultrafiltration, antagonistic activity was tested in both the membrane retentate and the filtrate.

RESULTS

Antagonistic activity of food-relevant filamentous fungi. Several strains of *P. nalgiovense*, *P. camemberti*, and *P. roqueforti* which were either taken from the culture collection of the Federal Research Centre for Nutrition, purchased as commercial starter cultures, or isolated from mold-fermented food products were tested for their ability to suppress the growth of *M. luteus* DSM 348. Positive fungal strains showed strong growth inhibition of *M. luteus* DSM 348 (Fig. 1). No inhibitory effect could be detected when strains of *P. roqueforti* and strains of *P. camemberti* were tested. In contrast, all but 1 of the 28 *P. nalgiovense* strains tested showed antagonistic activity in these experiments. Table 1 gives an overview of some selected examples of the analyzed strains. The antagonistic substance excreted by *P. nalgiovense* is relatively heat stable. Supernatants treated at 100°C for 20 min showed an activity of about one-half of that of the untreated samples. Ultrafiltration

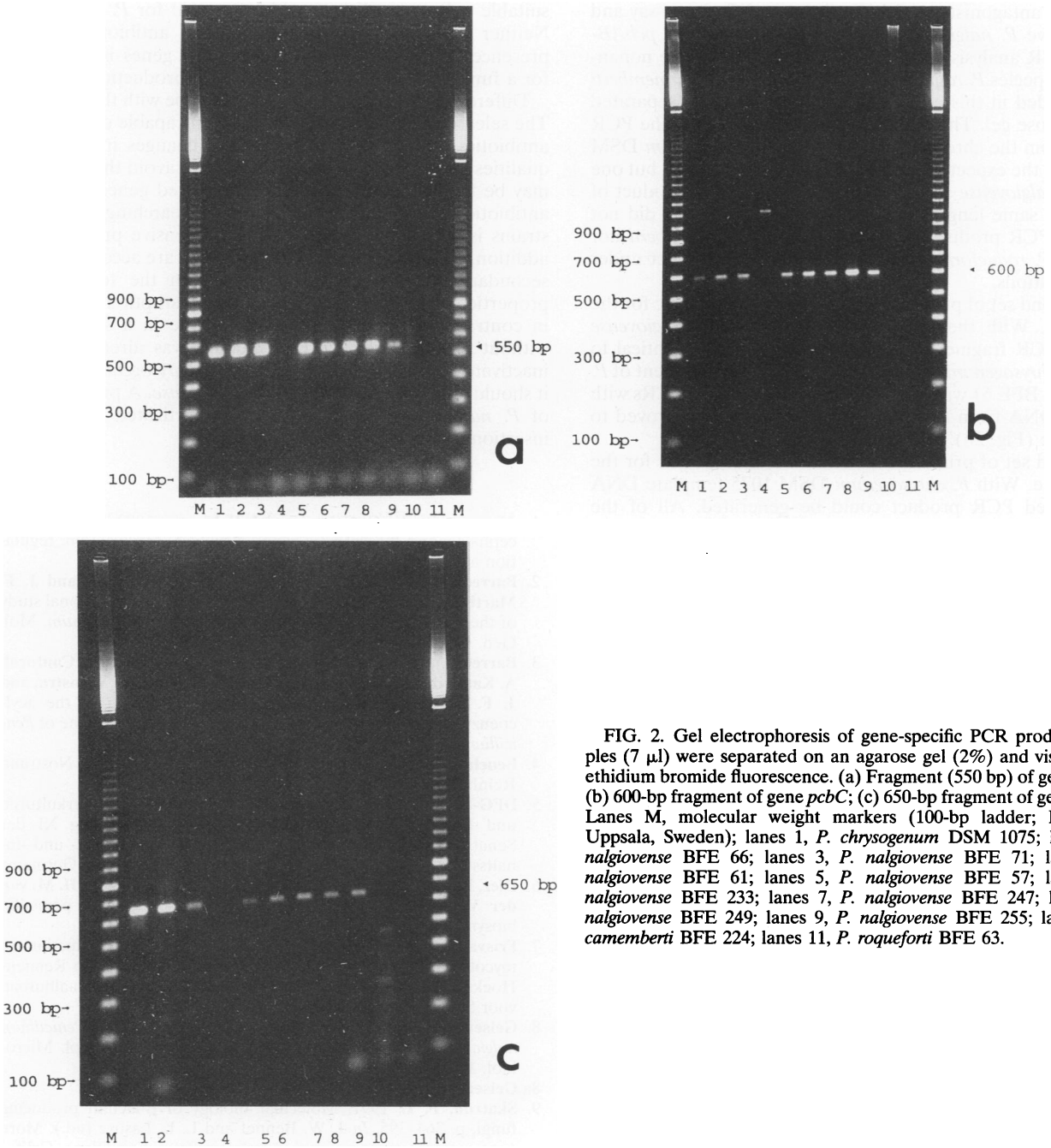

FIG. 2. Gel electrophoresis of gene-specific PCR products. Samples (7 μl) were separated on an agarose gel (2%) and visualized by ethidium bromide fluorescence. (a) Fragment (550 bp) of gene *pcbAB*; (b) 600-bp fragment of gene *pcbC*; (c) 650-bp fragment of gene *penDE*. Lanes M, molecular weight markers (100-bp ladder; Pharmacia, Uppsala, Sweden); lanes 1, *P. chrysogenum* DSM 1075; lanes 2, *P. nalgiovense* BFE 66; lanes 3, *P. nalgiovense* BFE 71; lanes 4, *P. nalgiovense* BFE 61; lanes 5, *P. nalgiovense* BFE 57; lanes 6, *P. nalgiovense* BFE 233; lanes 7, *P. nalgiovense* BFE 247; lanes 8, *P. nalgiovense* BFE 249; lanes 9, *P. nalgiovense* BFE 255; lanes 10, *P. camemberti* BFE 224; lanes 11, *P. roqueforti* BFE 63.

experiments using culture supernatants of submersed cultures indicated that the molecular weight of the antagonistic substance is below 1,000. These results suggest that *P. nalgiovense* is producing a secondary metabolite of low molecular weight. To get further information about the nature of this antagonistic substance, a volume of 300 μl of the supernatant was treated with 10 μl of penicillase (1.9 mg/ml) for 3 h at 37°C before being used in the agar diffusion assay. No inhibitory activity could be observed after this treatment (Fig. 1). These results indicate that *P. nalgiovense* is apparently capable of producing a β-lactam antibiotic.

Specific PCRs with the penicillin biosynthetic genes. Sequences of the three genes responsible for penicillin biosynthesis in *Penicillium chrysogenum* are known (2, 3, 6). The comparison of these sequences with the sequences of genes of other β-lactam-producing organisms revealed a striking homology even between nonrelated organisms (9). Because of this homology, primer sets which were synthesized according to the sequences of *P. chrysogenum* were used to confirm the presence of the three penicillin biosynthetic genes in *P. nalgiovense* by using PCRs under stringent hybridization conditions. Chromosomal DNA of a penicillin-producing strain of *P. chrysogenum* DSM 1075 was used as the template DNA in a control experiment. Thirteen strains of *P. nalgiovense* which

had shown antagonistic activity in the agar diffusion assay and one negative *P. nalgiovense* strain were subjected to *pcbAB*-specific PCR analysis. Additionally, five strains of the nonantagonistic species *P. roqueforti* and two strains of *P. camemberti* were included in this analysis. PCR products were separated on an agarose gel. The results are shown in Fig. 2a. The PCR product from the chromosomal DNA of *P. chrysogenum* DSM 1075 shows the expected fragment length of 550 bp. All but one of the *P. nalgiovense* strains analyzed show a PCR product of exactly the same length. Only *P. nalgiovense* BFE 61 did not show this PCR product. Neither strains of *P. camemberti* nor strains of *P. roqueforti* showed a specific PCR product under these conditions.

The second set of primers, ipns1 and ipns2, is specific for the *pcbC* gene. With the DNA of the inhibitory *P. nalgiovense* strains a PCR fragment with a molecular weight identical to that of *P. chrysogenum* was produced. The DNA fragment of *P. nalgiovense* BFE 61 was longer than the others. The PCRs with template DNA from *P. camemberti* or *P. roqueforti* proved to be negative (Fig. 2b).

The third set of primers, aat1 and aat2, was specific for the *penDE* gene. With *P. chrysogenum* DSM 1075 template DNA the expected PCR product could be generated. All of the analyzed *P. nalgiovense* strains except strain BFE 61 produced the same signal. Neither strains of *P. roqueforti* nor strains of *P. camemberti* showed specific positive results in this PCR (Fig. 2c).

DISCUSSION

It could be proved that *P. nalgiovense*, a food-related filamentous fungus, is able to produce penicillin. All strains analyzed showing antagonistic activity in the agar diffusion assay possess the genes *pcbAB*, *pcbC*, and *penDE*, encoding enzymes [δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine] synthetase, isopenicillin-*N*-synthase, and acyl coenzyme A:6-aminopenicillanic acid acyltransferase, which are essential in biosynthesis of the β-lactam antibiotic penicillin (Table 1). Only *P. nalgiovense* BFE 61 did not show any gene-specific DNA fragment in PCRs. Random amplified polymorphic DNA experiments specific for the species *P. nalgiovense* resulted in a different random amplified polymorphic DNA pattern when chromosomal DNA of strain BFE 61 was used as the template DNA. This suggests that this fungal strain morphologically classified as a member of *P. nalgiovense* belongs to a different RAPD genotype within this species (8a).

The other two food-related fungal species studied, *P. roqueforti* and *P. camemberti*, obviously do not carry genes specific for penicillin biosynthesis, suggesting that *P. nalgiovense* is more related to *P. chrysogenum* than to these two species.

Different principal requirements have to be fulfilled by fungal strains before the strains are used as starter cultures. One of these demands is the incapacity to synthesize metabolites with effects concerning human health. Therefore, the production of antibiotics has to be controlled (5). However, the existence of the complete set of genes necessary for penicillin biosynthesis and the production of a β-lactam antibiotic on a suitable culture medium could be proved for *P. nalgiovense*. Neither the ability to produce β-lactam antibiotics nor the presence of active penicillin biosynthetic genes is acceptable for a fungal starter culture used in food production.

Different approaches are possible to cope with this situation. The selection of new species which are incapable of producing antibiotics may result in fundamental changes in traditional qualities of mold-ripened foods. A way to avoid these changes may be the use of mutants with inactivated genes coding for antibiotic synthetic enzymes. However, searching for mutant strains is a time-consuming and cost-intensive procedure. In addition, the desired mutations frequently are accompanied by secondary mutations causing changes in the technological properties of the starter cultures. Gene disruption technology, in contrast, inactivates the targeted gene in a specific way, without any side effects. This method was already used to inactivate the *pcbC* gene in *Acremonium chrysogenum* (10), and it should also work in strains of *P. nalgiovense*. A protease gene of *P. nalgiovense* has already been inactivated by targeted insertion (8).

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