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Two *Penicillium camembertii* Mutants Affected in the Production of Cyclopiazonic Acid

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Penicillium camembertii **was mutated and screened for cyclopiazonic acid-negative mutants. With a simple and rapid mini-extraction method for detection of cyclopiazonic acid production, we were able to isolate two strains which were affected in the production of this metabolite. One strain had completely lost the ability to synthesize detectable amounts of this secondary metabolite, whereas the other mutant produced 50 to 100 times less cyclopiazonic acid than the wild type. Also, the former strain had a changed morphology compared with the wild type. This morphological alteration appears to be coupled to the inability to produce cyclopiazonic acid because morphological revertants were able to synthesize cyclopiazonic acid to a level comparable to the wild type. The second mutant accumulated a new metabolite which was detectable by two-dimensional thin-layer chromatography. This new metabolite, however, appears not to be a direct precursor of cyclopiazonic acid.**

Penicillium camembertii is a filamentous fungus which is important in food technology because it is used as a starter culture for the production of mold-fermented white cheese. Because of its lipolytic and proteolytic activity, it contributes to the flavor formation during the ripening of white cheese (8). However, this species is also able to produce cyclopiazonic acid (CA), a secondary metabolite toxic to animals and humans (9, 10, 12, 15). *P. camembertii* is able to produce this toxic metabolite in mold-fermented cheese (12) in various amounts, depending on the storage temperature (16). CA was not detected at the normal ripening temperature of 13°C; however, storage of the cheese at 25°C for 5 days resulted in detectable amounts of this toxin. The ability to produce CA is a common feature for *P. camembertii*. A total of 61 natural isolates tested for CA production showed positive results (5).

At the present time, nothing is known about the genes which are involved in the production of CA and their regulation. With the development of DNA-mediated transformation systems for *Penicillium* species (2–4, 7), it should be possible to isolate these genes by complementation (13) if mutants are available. In this work, we describe the isolation of mutant strains of *P. camembertii* which are affected in the production of CA.

MATERIALS AND METHODS

Chemicals. CA was purchased from Sigma. Radioactive tryptophan was obtained from NEN/Du Pont. The thin-layer chromatography (TLC) plates (Kieselgel 60) and all other chemicals were supplied by Merck.

Strains and growth conditions. *P. camembertii* Sp912 from the culture collection of the Federal Centre of Meat Research was used throughout this study. For determination of CA production, the fungus was grown at 25°C for 21 days on malt extract agar (48 g of malt extract agar [Merck] was dissolved per liter of water and autoclaved at 121°C for 15 min). The agar plates were covered with a sterile cellophane sheet (Ebert Folienwerk, Wiesbaden, Federal Republic of Germany) to avoid contamination with agar particles in the subsequent extraction procedure.

Mutation procedure. For the production of conidia from *P. camembertii*, the strain was grown on malt extract agar slants at 25°C for 7 days. The conidia were harvested by suspension in 5.4 ml of sodium acetate solution (0.1 M) to a concentration of approximately 10^5 spores per ml. A 0.6-ml portion of a 1 M $NaNO_2$ solution was added, and the suspension was kept at room temperature for 10 min. This resulted in a conidiospore survival rate of 10 to 15%. After this treatment, the suspension was plated on malt extract agar plates and incubated at 25°C for 4 days. Single colonies were picked, transferred to new plates, and incubated at 25°C for 21 days.

Determination of CA production. The determination of CA production by the mutated strains was carried out by TLC, as described by El-Banna et al. (5). For the extraction of CA, 0.2 g of mycelium (wet weight) was transferred to a microcentrifuge tube. A 500-μl portion of chloroform was added, and the tube was shaken for 10 min. The mycelium was removed, and the extract was evaporated to dryness in a Bachofer Speed Vac concentrator. The residue was resolved in 10 μl of chloroform and analyzed by TLC. The toxin was visualized by treatment of the TLC plates with 50% H_2SO_4 (vol/vol, in ethanol) and by subsequent spraying with a solution of 1% *p*-diaminobenzaldehyde in ethanol (Ehrlich reagent). After this treatment, the toxin became visible as a blue-purple spot (19).

Two-dimensional TLC. For two-dimensional TLC analysis of the chloroform-extractable compounds, baselines for each dimension were marked on the TLC plate. The two baselines formed a right angle, and at the crossing point the extract was loaded. Two spots of pure CA were applied as standard samples on the other ends of the two baselines. In the first dimension, the plate was chromatographed just below the application point of the standard sample for the second dimension, and vice versa.

Radioactive labeling of CA. The mutant Cpa2 and the wild type were grown for 6 days on minimal plates (minimal plates contained the following [in grams per liter]: glucose, 5;

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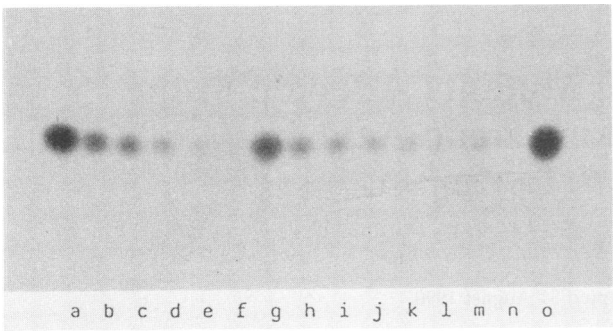

FIG. 1. Determination of CA production by the wild-type strain and the mutants Cpa1 and Cpa2. Different concentrations of pure CA (lanes a through f) and of the chloroform extract of the wild-type strain (lanes g through l) and the undiluted extracts from Cpa1 (lane n) and Cpa2 (lane m) were separated on a TLC plate. The amounts of CA loaded onto the plate were 6.5 (lanes a and o), 1.3 (lane b), 0.65 (lane c), 0.35 (lane d), 0.16 (lane e), and 0.11 (lane f) μg. The dilutions of the extract from the wild type were as follows: undiluted (lane g), 1:5 (lane h), 1:10 (lane i), 1:20 (lane j), 1:40 (lane k), and 1:60 (lane l). In each case 10 μl of the probe was applied on the TLC plate. As a solvent system, chloroform-isobutylmethylketone (4:1, vol/vol) was used.

KNO_3, 1.2; KH_2PO_4, 3.75; $MgSO_4$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1; KOH, 0.75; and agar, 15) and subsequently transferred to minimal plates containing 10 μM tryptophan and L-[5-^{3}H]tryptophan of a specific activity of 31.5 Ci/mmol at a concentration of 30 μCi/ml and incubated for 15 days at 25°C. The mycelia were harvested. The extraction procedure and the two-dimensional TLC analysis were performed as described above.

RESULTS

Screening for CA-negative mutants. For the screening procedure, we have developed a mini-extraction method which allows the screening of 144 mutated strains per TLC plate. Extraction of CA and application onto the TLC plate were carried out as a one-step procedure in a special device with disposable pipette tips (type, Gilson blue) as small extraction columns. Approximately 0.1 g of mycelium (wet weight) was transferred into the columns. The mycelium was wetted with 30 μl of chloroform. The chloroform extracted the CA and passed through the column onto the TLC plate, thereby producing a spot in a definite position. The plate was sprayed with the detection agent without previous separation of the spots. CA showed a specific blue-purple spot after this treatment. Negative results were confirmed with conventional extraction and TLC.

Isolation of mutants impaired in the production of CA. With the method described above, we were able to isolate mutants which were impaired in their ability to produce CA at a mutation frequency of 2×10^{-4}. One mutant, Cpa1, produced no detectable amounts of this toxin, whereas the other mutant, Cpa2, synthesized about 2% compared with the wild type (Fig. 1). The wild-type strain produced approximately 34 μg of CA per g of mycelium (wet weight) compared with 0.8 μg of this toxin produced by Cpa2. It could be observed, however, that this mutant accumulated a new metabolite within 21 days of incubation. On TLC plates the new compound appeared as a red spot when treated with the same detection reagents, compared with the blue-purple spot for CA. Both spots could be separated by two-dimensional TLC (Fig. 2).

Radioactive labeling of CA. Since it was possible that the new metabolite was a precursor of CA, a labeling experiment with [^{3}H]tryptophan was carried out. Tryptophan is the primary metabolite at the branch point to the secondary biosynthetic pathway for the production of CA (11). All precursors of CA after this branch point should be labeled by radioactive [^{3}H]tryptophan. Both the mutant Cpa2 and the wild type were grown on minimal medium containing labeled tryptophan. Because the mutant strain started to produce CA after 6 days of incubation (data not shown), 6-day-old colonies were transferred to minimal agar plates containing [^{3}H]tryptophan and incubated for 15 days further. To confirm that the mycelia of both strains had incorporated the labeled tryptophan under these conditions, parts of the mycelia were counted in a liquid scintillation counter. Both

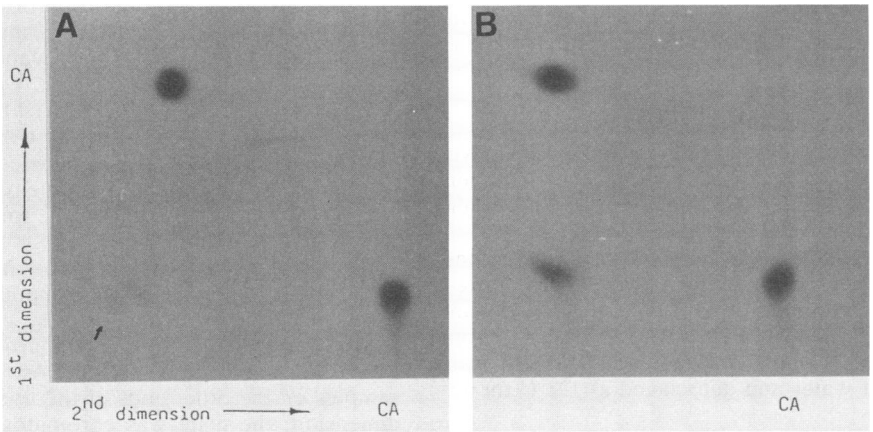

FIG. 2. Separation of the new accumulating metabolite in Cpa2 from CA by two-dimensional TLC. Chloroform-extractable compounds of Cpa2 (A) and the wild type (B) were analyzed by two-dimensional TLC (see Materials and Methods). In this way, the sample is chromatographed in two dimensions, whereas the two standard samples are chromatographed in one dimension only, indicating the position of CA in each dimension. The solvent system in the first dimension was chloroform-methanol (9:1), and in the second dimension it was chloroform-isobutylmethylketone (4:1). The new accumulating metabolite is indicated by an arrow.

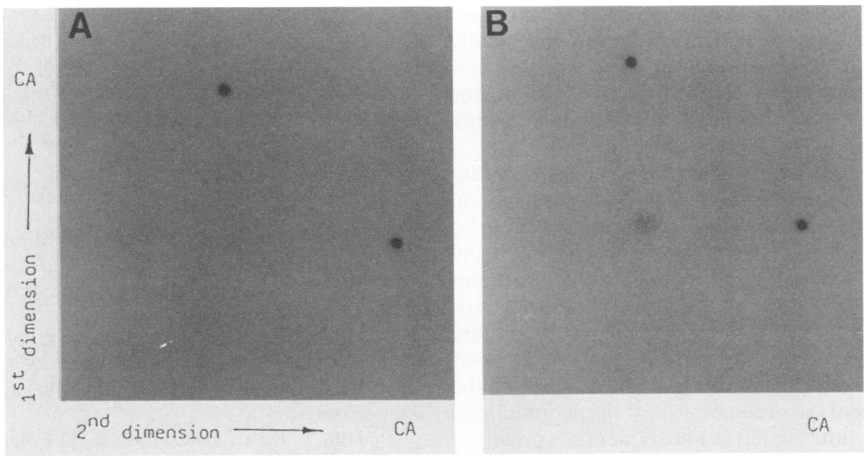

FIG. 3. Autoradiography of the two-dimensional TLC of the chloroform-extractable metabolites of the [^{3}H]tryptophan-labeled mutant Cpa2 (A) and wild-type (B) strains. The extraction of CA and the separation on TLC plates were described in Materials and Methods. Prior to the autoradiography, the TLC plates were sprayed with the detection reagent. The localization of the pure CA used as a control in each dimension was marked with 0.1 μCi of [^{3}H]tryptophan.

strains showed the same incorporation rate of about 10^5 cpm/mg of mycelium. Chloroform extracts of the mycelia were analyzed by two-dimensional TLC. The plates were sprayed with Ehrlich reagent. The amounts of CA and of the new metabolite produced on minimal medium were comparable to those produced on malt extract medium. Subsequently, the plates were subjected to autoradiography (Fig. 3). In the case of the wild type (Fig. 3B), the labeled CA gave a clear positive signal, indicating that the [^{3}H]tryptophan was incorporated into CA, as expected. However, no signal of a possible precursor of CA was detectable under the conditions used. These results suggest that there is no accumulation of any precursor metabolite in an amount comparable to the amount of CA within the wild type. In the case of the mutant Cpa2 (Fig. 3A), no signal is visible at all. Since CA is visible in the sprayed chromatogram (Fig. 2A), the incorporated radioactivity is below the detection limit of this method under these conditions. These results also give no evidence that a precursor metabolite of CA is accumulated as a major component of the cellular extract within the incubation time.

Because the new metabolite of Cpa2 accumulates only in small amounts, similar to that of CA (Fig. 2A), the results of this experiment don't exclude the possibility that this new metabolite is a precursor of CA. To solve this question, we have isolated the separated CA and the new metabolite by scraping out the spots and determined their radioactivity in a liquid scintillation counter. In Table 1 the results of this analysis are given. The highest activity can be found in the CA spot of the wild type. It has an activity nearly 100-fold that of the mutant strain, which confirms the results of the quantitative analysis (Fig. 1). The activity of the new metabolite, however, is in the range of the control, indicating that no radioactive tryptophan was incorporated into this substance.

TABLE 1. Radioactivity of the isolated extractable metabolites of the wild-type and the mutant strains

Substance	Radioactivity (cpm)	Strain
CA	19,328	Wild type
CA	221	Cpa2
New metabolite	66	Cpa2
Silica gel[a]	47	

[a] As a control, the radioactivity in approximately the same amount of silica gel was counted.

Mitotic stability of the isolated mutants. In addition to the lost ability to produce CA, Cpa1 showed a drastic change in morphology. It was almost completely unable to produce an aerial mycelium; however, it had the same growth rate as the wild type. This morphological alteration was found to be mitotically unstable. Approximately 10% of the colonies showed a segregation of the morphology into that of the wild type. After subculturing of these segregants, stable wild-type colonies were retained. Interestingly, these revertants had a capacity to produce CA comparable to that of the wild type.

In the case of Cpa2, the growth rate was slightly reduced compared with the wild type. However, the decreased ability to produce CA was mitotically stable, as shown by repeated subculturing of single-spore colonies on malt extract medium and subsequent analysis of CA production.

DISCUSSION

We have isolated two mutants of *P. camembertii* which show impaired production of CA. For the isolation of these mutants, it was essential to screen a large sample of mutated strains. For this purpose, we have developed a rapid mini-extraction method for the determination of CA production. This method is based on the specific staining of CA with Ehrlich reagent. CA-negative strains were easily detected without separation of the samples on the TLC plate. Benkhemmar et al. (1) have developed a screening procedure for the production of CA based on the antibacterial activity of this metabolite. With this method, however, it is not possible to clearly distinguish between CA producers and nonproducers. Filtenborg et al. (6) described the agar plug method for the rapid detection of intracellular mycotoxins. This method, however, has the disadvantage that contaminants from the agar lead to uncertain results if the samples are not separated on the TLC plate.

The isolated mutants Cpa1 and Cpa2 apparently had two different types of mutations. Cpa1 simultaneously showed a

drastic change in morphology and no detectable CA production. This phenotype, however, proved to be mitotically unstable. In each case, reversion of both phenotypic characteristics occurred simultaneously, indicating that the gene affected by the mutation is somehow involved in morphological development as well as in CA production.

Cpa2, however, is mitotically stable and shows no tendency for reversion, even after repeated subculturing of single spores. An additional metabolite could be detected by two-dimensional TLC. Labeling experiments with [^{3}H]tryptophan, however, revealed that this metabolite had not integrated tryptophan and that no other potential precursor of CA accumulated to the point of being a major component within the mutant strain. The described experiments, however, did not exclude the possibility that in the case of the wild type or the mutant, a precursor may be accumulated during the incubation time which is subsequently converted to CA in the case of the wild type or to another metabolite which is not extractable with the solvent system used in the case of the mutant.

The isolation of these mutants, especially Cpa2, which apparently has a mutational block in the biosynthetic pathway from tryptophan to CA, may enable the isolation of the particular gene by complementation of the mutation.

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