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Abstract

Penicillium camembertii is a filamentous fungus used for the production of mold- fermented white cheese. It is a domesticated form of *Penicillium commune*, especially adapted to the food environment (Pitt et al. 1986. Food Microbiol. 3:363-371). Despite its use as food starter culture, *P. camembertii* is able to produce cyclopiazonic acid (CA), a secondary metabolite toxic to animals and humans (Holzapfel 1968. Tetrahedron 24:2101- 2119); Le Bars 1979. Appl. Environ. Microbiol. 38:1052-1055). The synthesis of CA starts from the amino acid tryptophan with the condensation of acetyl-CoA and isoprenoids, especially dimethyl allylpyrophosphate (Holzapfel 1980. In: P.S. Steyn, The biosynthesis of mycotoxins, Academic Press). Acetyl-CoA is also the direct precursor of the isoprenoids

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Penicillium camembertii is a filamentous fungus used for the production of mold- fermented white cheese. It is a domesticated form of *Penicillium commune*, especially adapted to the food environment (Pitt et al. 1986. Food Microbiol. 3:363-371). Despite its use as food starter culture, *P. camembertii* is able to produce cyclopiazonic acid (CA), a secondary metabolite toxic to animals and humans (Holzapfel 1968. Tetrahedron 24:2101- 2119); Le Bars 1979. Appl. Environ. Microbiol. 38:1052-1055). The synthesis of CA starts from the amino acid tryptophan with the condensation of acetyl-CoA and isoprenoids, especially dimethyl allylpyrophosphate (Holzapfel 1980. In: P.S. Steyn, The biosynthesis of mycotoxins, Academic Press). Acetyl-CoA is also the direct precursor of the isoprenoids.

To define the genes responsible for the production of cyclopiazonic acid, *P. camembertii* Ps 912 was treated with nitrous acid and screened for CA negative mutants. One mitotically stable strain was isolated with only 1-2% of CA production compared to the wild type (Geisen et al. 1990. Appl. Environ. Microbiol. 56:3587-3590).

To localize the mutation either in the pathway for biosynthesis of isoprenoids or in the biosynthetic branch between tryptophan and CA, radioactive labelling either with 3H- tryptophan or with 14C-acetate was carried out. For this purpose *P. camembertii* was grown on minimal medium (per liter: 5 g glucose, 3.75 g KH_2PO_4, 0.5 g $MgSO_4$, 0.1 g NaCl, 0.1 g $CaCl_2$, 0.75 g KOH, 1.2 g KNO_3, 15 g agar) for 5 days at 25 C and transferred to minimal medium containing either L-[5-3H]-tryptophan (at a concentration of 30 uCi/ml with a specific activity of 31.5 Ci/mmol) or [14C]-acetic acid, sodium salt (at a concentration of 10 mCi/ml with a specific activity of 59 mCi/mmol), and grown at 25 C for 5 days. After that time the same amount of cell material either from the mutant strain or from the wild-type strain was recovered from a colony, transferred to a microcentrifuge tube and treated with 1 ml chloroform for 5 min in a microcentrifuge shaker. The mycelium was discarded and the chloroform evaporated in a vacuum centrifuge. The residue was redissolved in 10 ul chloroform and applied to a thin layer chromatography plate (Kiesel-gel 60, Merck, Darmstadt) and chromatographed in two dimensions (solvent systems: first dimension, chloroform/methanol:9/1; second dimension chloroform/isobutylmethylketone:4/1). The Rf values were determined by dividing the migration length of the respective spot by the migration length of the solvent. After chromatographic separation the plates were autoradiographed.

The mutant and the wild-type strain were first labelled with 3H-tryptophan. If there is no regulatory feedback mechanism to prevent an accumulation of the respective precursor there should be an accumulation of a new metabolite just "in front" of the mutation if the mutation is

located within the biosynthetic branch between tryptophan and CA. The results of the experiment are shown in Figure 1. Beside the clear signal of CA in the case of the wild type, there was no other chloroform extractable 3H-tryptophan-labelled material under the conditions used. In the case of the mutant strain, there was no signal at all, indicating the mutant had not integrated radioactively labelled tryptophan into extractable precursors of CA. These results indicate the mutation is probably not located within this biosynthetic pathway, assuming there is no regulatory feedback mechanism. The fact that the mutant strain was also able to grow on minimal medium without tryptophan indicates the mutation has not affected the pathway for biosynthesis of this amino acid.

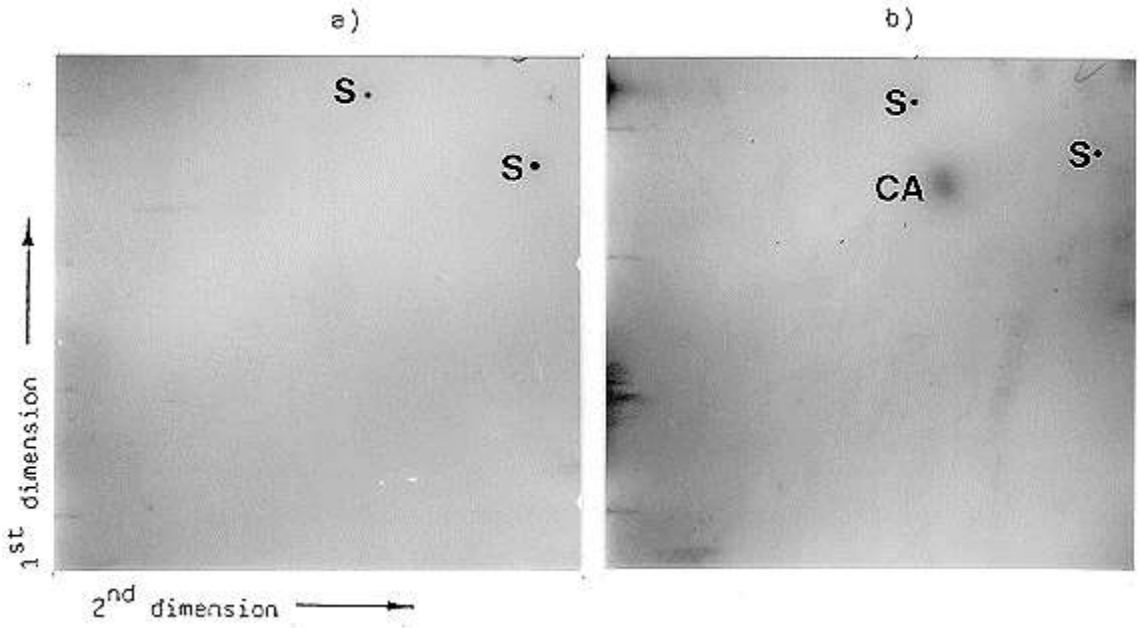

Figure 1. Autoradiography of the two-dimensional thin layer chromatography of the chloroform-extractable metabolites of the [3H]tryptophan-labelled mutant (a) and the wild type (b). The extraction of CA and the solvent systems used in each dimension are described in the text. For two-dimensional thin layer chromatography, baselines for each dimension were marked on the thin layer 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. The localization of pure CA used as control in each dimension was marked with 0.1 uCi of [3H] tryptophan. Standard samples = s, extracted cyclopiazonic acid = CA

To test the possibility that the mutation has influenced the pathway for isoprenoid biosynthesis, the mutant and the wild-type strain were grown on minimal medium containing 14C-acetate. Acetate is the precursor of the isoprenoids, so labelling with 14C-acetate should give different patterns of extractable metabolites if the mutation is located within this pathway. Figure 2 shows the result of this experiment. There is a different signal pattern between the mutant (Fig. 2a) and the wild type (Fig. 2b). In the case of the mutant there are clearly differences in the separation of the spots on the baseline of the first dimension. These spots are not able to migrate by using the second solvent system. A clear resolution of one spot in the second dimension at a higher Rf

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value (0.868) could be observed in the case of the mutant (Fig. 2a, 1). In the case of the wild type, resolution of two spots occurred (Fig. 2b, 2 and 3). The solvent system used in the second dimension shows poor separation capacity for these substances. The fact that the Rf values are different in the mutant compared with the wild type (0.723 and 0.568) indicates different labelled substances. The results show that the mutation affects different chloroform extractable metabolites labelled with 14C-acetate rather than affecting a single substance. Similar complex changes of the labelling pattern of the mutant compared to that of the wild type could be observed when the strains were incubated for a longer period of time (14 days or 21 days) on medium containing 14C-acetate (data not shown). In these cases the overall patterns from both the mutant and the wild type were different from the patterns seen in Fig. 2, indicating a change of the accumulated labelled metabolites during the time course of incubation. These results show that the mutation leads to complex changes in the pattern of the 14C-acetate-labelled metabolites and suggests it is located in the pathway for isoprenoid biosynthesis.

Alternative possibilities that condensation of acetyl-CoA residues to tryptophan is abolished or that the mutation affected the pathway from tryptophan to CA, and that an accumulation of a precursor of that pathway is prevented by a feedback mechanism are not likely because in any case the 14C-acetate labelling pattern should not be changed.

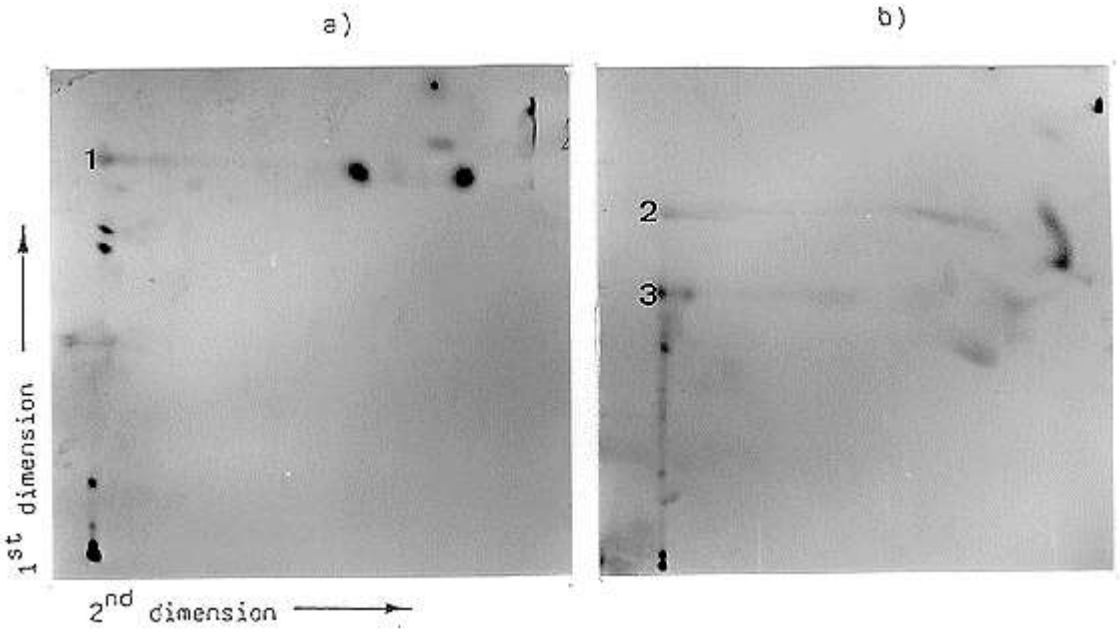

Figure 2. Autoradiography of the two-dimensional thin layer chromatography of the chloroform-extractable metabolites of the [14C]acetate-labelled mutant (a) and the wild-type strain (b). Extraction of CA and the solvent system of each dimension are described in the text. 1,2,3 = separable spots by using the second solvent system.