Heterokaryosis between *Aspergillus oryzae* Cyclopiazonic Acid-Defective Strains: Method for Estimating the Risk of Inducing Toxin Production among Cyclopiazonic Acid-Defective Industrial Strains

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*Aspergillus oryzae* strains are used extensively in the food industry. Some of these strains excrete α-cyclopiazonic acid (CPA), a mycotoxin which may provoke toxicoses in rats. Physicochemical methods may reveal the presence of this toxin, but they are inadequate to screen CPA-nonproducing (CPA') strains. CPA production is revealed by either bacterial growth inhibition or alkalization of the culture medium. This first biological property was used to devise a time-saving screening method to isolate mutants affected in their ability to produce CPA. The second method was used as a further test. After N-methyl-N'-nitro-N-nitrosoguanidine treatment, we isolated CPA'-mutants from CPA producer strains (CPA') and CPA' mutants from CPA' strains. The mutants unable to produce CPA may be used in the food industry to reduce or eliminate the risk of intoxication in humans. Heterokaryon formation between different mutant strains was carried out to evaluate the risks of obtaining CPA from a mixture of mutants modified in their ability to synthesize this toxin. Pairings between two CPA' strains always gave rise to CPA' heterokaryons. Pairings between CPA' and CPA' strains led, most often, to CPA' heterokaryons. This could be directly correlated to the more frequent genotype (CPA') in the heterokaryon. CPA hypoproducer and hyperproducer heterokaryons were obtained. Pairings between CPA' strains always gave rise to CPA' heterokaryons. These results suggest that the risks of producing this toxin from two CPA' individuals are not high.

The mold species *Aspergillus oryzae* plays an important role as a starter in the food technology industry because of its ability to produce considerable quantities of amylolytic, proteolytic, and lipolytic enzymes. However, *A. oryzae* also produces numerous metabolites that are toxic to humans and certain animals (12, 14).

A study of mycotoxins produced by 16 *A. oryzae* strains used in the food industry revealed that 8 strains produced a mycotoxin: α-cyclopiazonic acid (CPA) (15).

CPA is a derivative of tryptophan and exhibits a 3-acetyltetramic structure. This mycotoxin induces toxicoses in rats (17). Recently, Sorensen et al. (20) have revealed that CPA has mutagenic activity in *Salmonella typhimurium*.

To avoid any risk of intoxication to humans, the use in the food industry of strains screened for their inability to produce CPA (CPA') appears to be a prerequisite.

Without any screening method, the selection of CPA'-mutants requires analysis of a great number of strains; each strain must be tested either through one of three biological assays generally used to reveal the toxicity of fungal metabolites (growth inhibition in chick embryos, inhibition of the growth of rat-hepatoma cells in vitro, and acute toxicity in mice [12]) or through the chemical analysis of culture filtrates.

As many mycotoxins exhibit antibacterial activity (1, 5, 18, 19), we investigated its presence in CPA.

A correlation between this activity and the production of CPA was shown. Furthermore, pH variations in the culture medium during the production of CPA were also investigated. This first property was used to devise a simple screening method to isolate, after N-methyl-N'-nitro-N-nitrosoguanidine treatment, mutants affected in their ability to synthesize CPA, particularly CPA-defective strains.

The industrial use of such strains may limit risks of intoxication in humans. However, this precaution may prove to be inefficient. As a matter of fact, industrial methods often involve the use of a mixture of strains exhibiting complementary characteristics. Metabolic exchanges via the substrate or the formation of anastomoses between vegetative filaments, which may allow exchanges of genetic information, may reinstate CPA synthesis.

The purpose of this study was to evaluate the risks of obtaining CPA synthesis from heterokaryons between mutants affected in their ability to produce CPA.

**MATERIALS AND METHODS**

**Strains.** *A. oryzae* 102.07 and 115.33 were obtained from the Centraal Bureau Voor Schimmelcultures in Baarn (Holland). Strain 102.07 has been described previously by Orth (15) as a CPA producer strain (CPA'), and strain 115.33 has been described as a non-CPA producer (CPA').

**Culture media.** Potato dextrose agar (PDA) was used to grow cultures.

A minimum mineral medium (Mm), consisting of glucose (5
g); NO₃K (1.2 g); KH₂PO₄ (3.75 g); MgSO₄ · 7H₂O (0.5 g); NaCl (0.1 g); CaCl₂ (0.1 g); KOH (0.75 g); Bacto-Agar (20 g; Difco Laboratories) per liter of medium, adjusted to pH 6.2, was used to select prototrophic fusion products and to characterize the auxotrophic mutants. CPA was produced on the M medium (M₁) described by Holzapfel and Wilkins (8). Because auxotrophic mutants do not grow on M₂, toxin production was assessed on M₂ supplemented with the required growth factor.

For bacterial growth, LB medium was used (Bacto-Peptone 10 g/liter [Difco]; yeast extract [5 g/liter]; NaCl [5 g/liter]; agar [7 g/liter]).

**CPA production.** Unshaken liquid cultures were used to produce CPA. Erlenmeyer flasks (250 ml) containing 75 ml of M₂ were inoculated with about 10⁶ conidia from a single-spore culture and incubated at 25°C for 21 days.

**CPA extraction.** CPA was extracted from culture filtrates by a modified Le Bars (13) technique. A 40-ml portion of culture filtrate, adjusted to pH 3 with aqueous HCl (50:50), was extracted with four volumes of CH₃OH-CHCl₃ (1:4). The CH₃OH-CHCl₃ layer was decanted and retained, and then it was dried with Na₂SO₄, filtered, and vacuum concentrated to dryness.

The crude extract was taken up in CHCl₃ for characterization.

**CPA characterization.** Crude extracts were characterized by thin-layer chromatography (TLC) and by nuclear magnetic resonance (¹H-NMR) analysis. A purified CPA extract was used as a control. TLC was performed on silica gel 60F254 that had been previously impregnated with oxalic acid (0.4 M) and dried. Elution was performed in isobutyrylmethane-ketone-chloroform (1:4). CPA was visualized as a blue-violet spot in ordinary light after it was sprayed with Ehrlich reagent (p-dimethylaminobenzaldehyde 10% [wt/vol] in HCl-acetone [1:4]).

¹H-NMR spectra (Perkin-Elmer R32 90-MHz spectrometer) of extracts taken up in 0.5 ml of deuterochloroform were performed. Chemical shifts (δ) were expressed in parts per million (10⁻⁶) by reference to the tetramethylsilane peak. Quantitative analysis was performed by introducing into crude extracts a known concentration of CH₃NO₂ solubilized in chloroform. The methyl group of CH₃NO₂ shows a singlet peak at δ = 4.33 ppm, which is different from the singlet peak of CPA (δ = 2.49 ppm). The peak height ratio was used to quantify the toxin present in each extract with a precision of 10%.

**Biological methods.** Antibacterial activity of A. oryzae culture filtrates was revealed in the presence of two antibiotic-sensitive bacterial strains: Escherichia coli HFRG6 and Bacillus subtilis 168. For the disk assay, petri dishes (90 mm in diameter) were filled with 20 ml of semisolid LB medium at 40°C inoculated with the bacterial suspension (10⁶ cells). LB medium was supplemented with 0.2 g of MgSO₄ per liter in the case of B. subtilis 168. Paper disks (6 mm in diameter) impregnated with 15 μl of the various culture filtrates and crude extracts were placed on the surface. Plates were incubated at 37°C for 24 h. Activity of CPA was expressed as the diameter of the inhibition zone.

Bacteriotoxic effects of substances secreted in the culture medium may be tested in situ by the (semipermeable) cellophane membrane culture technique. The fungus may grow normally on the surface of such a membrane by taking up nutrients in the medium through the cellophane, and conversely, it may secrete its toxins into the medium. After the membrane and the growing culture were peeled off, the presence of bacterial growth-inhibiting metabolites was revealed by filling its place 10 ml of a semisolid LB medium at 40°C inoculated with a bacterial culture. Plates were read after incubation for 24 h at 37°C.

**Selection of mutant strains.** Conidia from young thalli (3 to 5 days old, depending on the strain) were plated on fresh PDA. After 4 days of incubation, several young conidia were picked up and isolated. After 4 days of incubation, small fragments were taken from each colony and transferred to PDA and to M₃. Clones unable to grow on M₁ were retained, and their auxotrophy was determined by the method of Holliday (6).

To select CPA-modified mutants, a screening method was devised especially for this study. After mutagenesis, diluted conidial suspensions were sprayed onto M₂ covered with a cellophane membrane. Antibacterial activity of growing thalli was characterized in situ.

**Balanced heterokaryons.** We used the method of heterokaryon formation described by Dabousi-Bareyre (3). Heterokaryons were analyzed by sampling conidia and hyphal fragments containing no more than three cells. Typical conidia of A. oryzae contain between one and six nuclei. During conidial formation, several nuclei may migrate together from the vesicle toward the conidium, and each individual nucleus undergoes an independent mitosis (10).

**Protoplast production.** Protoplasts were obtained by the method of Malard (Ph.D. thesis, Université des Sciences et Techniques de Lille, France, 1981). A total of 100 mg of 24-h-old mycelium was incubated for 5 h at 23°C and shaken gently in the presence of the following lytic complex: cytohelicase (5%; Industrie Biologique Française); phosphate buffer (0.1 M, pH 6); HCl (0.6 M); citric acid (0.05 M). Protoplasts were collected after filtration and then washed by successive centrifugation in phosphate buffer (0.1 M; pH 6)–KCl (0.6 M). Protoplasts were counted in a hemocytometer, and their regeneration rate was controlled on PDA–30% saccharose.

**Protoplast fusion.** By the technique of Malard (Ph.D. thesis), 10⁶ protoplasts from each of the two strains to be fused were centrifuged for 15 min at 3,000 rpm and then incubated for 15 min at 30°C in the following solution: polyethylene glycol 30% (PM 6000), CaCl₂ (0.015%), glycerol (0.05 M), adjusted to pH 7.5 with NaOH (0.1 M). After incubation, one volume of the suspension was diluted in five volumes of NaCl (0.6 M) and centrifuged for 15 min at 3,000 rpm. The protoplasts were finally suspended in 4 ml of NaCl (0.7 M). Serial dilutions were plated on solid M₁ medium–30% saccharose to select nutritionally complementing fused protoplasts. As a control, protoplasts of each mutant strain were plated on solid M₁ medium–30% saccharose. The number of viable protoplasts in each strain was determined by plating on solid PDA–30% saccharose. All plates were incubated at 23°C.

**RESULTS**

**Selection of CPA⁻ mutants.** (i) Production of CPA by A. oryzae wild-type strains. TLC analysis of purified CPA and of several crude extracts of CPA⁺ strain 102.07 revealed a blue-violet spot at R₂ = 0.75. On the other hand, crude extracts of CPA⁻ strain 115.33 and those from the uninocu-
labeled M medium never did reveal this spot. $^1$H-NMR analysis confirmed these results.

The control spectrum of 0.6 mg of purified CPA showed several peaks. Identification of the peak characteristics of the various chemical groups constituting CPA was realized by comparison with those described by Holzapfel (7) (Fig. 1).

The spectrum contained seven signals, each of which corresponded to a particular group of the CPA molecule (Table 1).

$^1$H-NMR spectra of crude extracts of strain 102.07 (CPA⁺) showed four peaks characteristic of CPA; the others were shadowed by contaminating molecules, particularly the peak characteristic of the indole nucleus (Table 1).

$^1$H-NMR spectra of crude extracts of strain 115.33 (CPA⁻) and of an uninoculated M₂ medium extract did not show any characteristic peaks of CPA.

(ii) CPA biological properties. The disk assay was used to study the antibacterial activity of CPA contained either in crude extracts or directly in culture filtrates.

Purified CPA was used as a control. With both E. coli and B. subtilis, culture filtrates and crude extracts of strain 102.07 showed growth inhibition zones from 6 to 8 mm in diameter around the paper disk, which were similar to those obtained in the control. On the contrary, in most cases culture filtrates and crude extracts of strain 115.33 and of uninoculated M₂ medium did not show growth inhibition zones. In a few cases, a slight inhibition was noted in strain 115.33, which may have been due to other toxic metabolites excreted by the fungus.

We took advantage of this last observation and of the cellophane membrane technique to devise a method which characterizes CPA production directly from the medium. This method is described in Fig. 2.

The following observations were made. (a) The growth of E. coli and B. subtilis was clearly inhibited in zones in which the CPA⁺ strain (102.07) previously grew. (b) The growth of E. coli and B. subtilis was, in most cases, not affected in zones in which the CPA⁻ strain (115.33) previously grew. However, in a few instances, a weak inhibition was noted, but it was always in small central areas.

These results confirm those obtained by the disk assay.

The positive correlation between bacterial growth inhibition and the presence of CPA in the culture medium, and conversely, between normal bacterial growth and the absence of CPA, was used as a screening method to isolate mutants affected in their ability to produce CPA.

In the course of this study, we noticed that the pH of unshaken cultures of strain 102.07 (CPA⁺) on M₂ medium increased from 5.5 to 8.5 after 21 days, whereas the pH of strain 115.33 (CPA⁻) varied from 5.5 to 6.5. We thus added a pH indicator (phenol red: yellow at pH 6.8 and red at pH 8.4) to M₂ medium. This substance does not affect the growth of A. oryzae. After 8 to 10 days of growth on this

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**TABLE 1. CPA characteristic peaks in $^1$H-NMR spectra of purified CPA and crude extracts of strain 102.07**

<table>
<thead>
<tr>
<th>Nature of the peak</th>
<th>Corresponding chemical group</th>
<th>8 (ppm) in:</th>
<th>Purified CPA</th>
<th>Extract of strain 102.07</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doublet</td>
<td>C(21)H₃ and C(22)H₂</td>
<td>1.61</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>Singlet</td>
<td>C(20)H₄</td>
<td>2.49</td>
<td>2.47</td>
<td></td>
</tr>
<tr>
<td>Doublet</td>
<td>C(12)H₂</td>
<td>3.04</td>
<td>3.03</td>
<td></td>
</tr>
<tr>
<td>Quadruplet</td>
<td>C(4)H₄</td>
<td>3.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doublet</td>
<td>C(5)H₃</td>
<td>4.04</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>Triplet</td>
<td>Indole</td>
<td>7.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singlet</td>
<td>N(1)H</td>
<td>8.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Carbon number according to Holzapfel (7).
medium, strains 102.07 and 115.33 induced in the medium a red and a yellow color, respectively.

We used this property to confirm the results obtained by the antibacterial screening method.

(iii) Isolation of mutants affected in their ability to produce CPA. Using the antibacterial screening method, we isolated, with a frequency of $3 \times 10^{-4}$, CPA- mutants from strain 102.07 (CPA+) named TB500 and 32.1. It is noteworthy that we also obtained a mutant (T50) which produced more CPA than did strain 102.07.

This suggests that a wide variety of mutants affected in their ability to produce CPA could be randomly obtained.

Thus, from a CPA- strain (115.33) we obtained two CPA+ mutants with a frequency of $4 \times 10^{-4}$.

The CPA production of all these mutants was controlled by TLC and $^1$H-NMR.

Table 2 shows the $^1$H-NMR spectra characteristics of these various mutant crude extracts, and the quantity of CPA (in micrograms per milliliter of filtrate) which they produced.

**Genetic analysis through heterokaryosis. (i) Selection of auxotrophic mutants.** We isolated several auxotrophic mutants from CPA- strains (115.33, TB500, and 32.1) and from a CPA+ strain (102.07). Their conidial germination rate and their ability to produce CPA were controlled. These mutants are described in Table 3.

(ii) Selection of balanced heterokaryons. Reciprocal nutritional complementation through the medium was never observed in paired auxotrophic mutants.

The pairing results are as follows (Fig. 3): (a) Of 62 pairings, 26 formed prototrophic sectors. Among these 26 pairings, 16 resulted from pairings between mutants issued from the same parental genotype (15 between 102.07 mutants, 1 between 102.07 mutants, and 1 between 115.33 mutants), and only 10 resulted from pairings between mutants issued from different genotypes (102.07 $\times$ 115.33).

(b) Of 62 pairings, 32 did not form prototrophic sectors. For some of these pairings we isolated protoplasts and fused them. We never obtained prototrophic individuals.

(iii) Analysis of prototrophic products. In the prototrophic cultures, the analysis of a sample of about 200 monokonidia always revealed large amounts of auxotrophic parental genotypes. In all cases, one of the genotypes was favored over the other. This advantage may reach 95%.

It is noteworthy that within a particular prototrophic thallus, the frequency of one parent may be either high or low, depending on the frequency of other partner. For instance, asn (32-1B) is found with a frequency of 86% in a pairing with trp (32.1) and of 12% with PAB.

In these 200 isolates, 1 to 2% of the conidia were prototrophic. The conidia gave rise to thallus which again segregated both parents by the same ratios. This was shown to be the case until the sixth subcloning. Since A. oryzae conidia are plurinucleated, these results strongly support the heterokaryotic nature of the prototrophic conidia.

Hyphal fragment analysis revealed an identical phenomenon (Table 4).

(iv) Influence of heterokaryosis on CPA production. With both biological methods, the ability of each heterokaryotic thallus to produce CPA was determined. The results may be summarized as follows. (a) Pairings between two CPA+ auxotrophic strains all issuing from strain 102.07 gave rise only to CPA+ heterokaryons. (b) Six pairings performed between one CPA+ and one CPA- auxotrophic strain gave

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**TABLE 2. $^1$H-NMR spectra characteristics of mutants**

<table>
<thead>
<tr>
<th>Characteristic peaks of CPA (8 [ppm])</th>
<th>Original strains</th>
<th>Mutant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>102.07 (CPA+)</td>
<td>102.07 Induced from 102.07</td>
</tr>
<tr>
<td></td>
<td>T50  TB500  32.1  NPP7  450</td>
<td></td>
</tr>
<tr>
<td>C(21)$^4$H$_2$ (1.61)</td>
<td>+     0     +    0</td>
<td>+     0     0     +    0</td>
</tr>
<tr>
<td>C(22)H$_2$ (1.61)</td>
<td>+     0     +    0</td>
<td>+     0     0     +    0</td>
</tr>
<tr>
<td>C(20)H$_2$ (2.49)</td>
<td>+     0     +    0</td>
<td>+     0     0     +    0</td>
</tr>
<tr>
<td>C(12)H$_2$ (3.04)</td>
<td>+     0     +    0</td>
<td>+     0     0     +    0</td>
</tr>
<tr>
<td>C(4)H (3.50)</td>
<td>0     0     0    0</td>
<td>0     0     0     0    0</td>
</tr>
<tr>
<td>C(5)H (4.04)</td>
<td>+     0     +    0</td>
<td>0     0     0     0    0</td>
</tr>
<tr>
<td>Indole (7.21)</td>
<td>0     0     +    0</td>
<td>0     0     0     0    0</td>
</tr>
<tr>
<td>N(1)H (8.16)</td>
<td>0     0     0    0</td>
<td>0     0     0     0    0</td>
</tr>
<tr>
<td>Quantity of CPA (µg/ml of filtrate)</td>
<td>7.1    0     9.7  0.75</td>
<td></td>
</tr>
</tbody>
</table>

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* a + , peak; 0, no peak.
* b Carbon number according to Holzapfel (7).
rise, in five cases, to CPA\(^+\) heterokaryons. Only one exception was noted in which the heterokaryon issued from the pro \((102.\text{A}) \times nd\) pairing did not produce CPA. (c) Of the 17 pairings between two CPA\(^-\) auxotrophic strains from the same genotype or from different genotypes, CPA\(^-\) heterokaryons were produced (Table 4).

It is interesting that a mixed culture of two prototrophic CPA\(^-\) strains (all possible pairings among 115.33, 32.1, and TB500 were tested) never revealed any CPA production.

\(^1\)H-NMR analysis allows a finer detection of the toxin and confirms these results.

In pairings between CPA\(^+\) and CPA\(^-\) strains, \(^1\)H-NMR quantitative analysis revealed two classes of heterokaryons: (a) those which produce less CPA than the producing parent \((\text{met} \times \text{asn})\) and \((\text{met} \times nd\) heterokaryons), and (b) those which produce more CPA than the producing parent \((\text{fol} \times PAB\) and pro \((102.\text{B}) \times PAB\) heterokaryons). Table 5 shows the results for some pairings.

### DISCUSSION

The use of *A. oryzae* in the food industry involves a risk of intoxication for humans or animals, because several strains have been shown to produce CPA. The selection of strains unable to produce CPA, either from wild types of *A. oryzae* strains or after mutagenesis, may reduce or eliminate this risk.

Ordinarily, the selection of this type of mutant requires the analysis of large samples. In this case classical investigation methods (physicochemical techniques) are inadequate since they require lengthy experiments. However, these same techniques may be quite useful to perform in-depth analyses of small, preselected samples.

TLC allows the quick detection of CPA in crude extracts. Strain 102.07 reveals a blue-violet spot with \(R_f = 0.75\), an indication of CPA production \((7, 13)\), whereas strain 115.33 does not reveal this spot. These results confirm those of Orth \((15)\).

A more precise analysis by \(^1\)H-NMR provides more convincing proof of the production or nonproduction of CPA by *A. oryzae* strains. Taking into account the sensitivity of this method, we assert that strain 102.07 produces CPA and that strain 115.33 does not.

Such analyses are required to obtain qualitative and quantitative data on our strains, but are inefficient in selecting mutants affected in the ability to produce CPA. The effect of CPA on bacterial growth was used to devise a method based on a selection criterion easily revealed in vitro. The pH variation of the medium during CPA synthesis is a complementary property which reduces the risk of errors inherent to the sensitivity of the first method. Indeed, minute amounts of CPA may not always be detected.

Nevertheless, these techniques allow the selection of CPA\(^-\) mutants of *A. oryzae* which do not involve any risk to the health of consumers. They also allow the selection of CPA\(^+\) mutants from CPA\(^-\) strains with the same frequencies. We also detected a CPA hyperproducer mutant from a CPA\(^+\) strain.

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**TABLE 3.** Auxotrophic mutants and their characteristics

<table>
<thead>
<tr>
<th>Origin</th>
<th>Metabolic requirements for growth on M(_i)</th>
<th>Designation</th>
<th>Conidial germination on PDA (%)</th>
<th>CPA production</th>
</tr>
</thead>
<tbody>
<tr>
<td>115.33</td>
<td>Asparagine ((\text{asn}))</td>
<td>nd</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>(CPA(^-))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>102.07</td>
<td>Methionine ((\text{met}))</td>
<td>pro ((102.\text{A}))</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>(CPA(^+))</td>
<td>Proline ((\text{pro}))</td>
<td>pro ((102.\text{B}))</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Tryptophane ((\text{trp}))</td>
<td>hisB1</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Vitamin B(_1)</td>
<td>fol</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>32.1</td>
<td>Folic acid ((\text{fol}))</td>
<td>asn ((32.1 \text{A}))</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td>(CPA(^-))</td>
<td>Tryptophane ((\text{trp}))</td>
<td>asn ((32.1 \text{B}))</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid and vitamin B(_6)</td>
<td>gluB6</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>TB500</td>
<td>Paraamino-benzoic acid ((\text{PAB}))</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 4.** Prototrophic thalli analysis of the rate of parental and prototrophic genotypes and CPA production in prototrophic thalli

<table>
<thead>
<tr>
<th>Pairings (genotype 1 (\times) genotype 2)</th>
<th>Conidia (%) with the following characteristics:</th>
<th>Hyphal fragments (%) with the following characteristics:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prototrophic Genotype 1</td>
<td>Auxotrophic Genotype 2</td>
</tr>
<tr>
<td>CPA(^+) (\times) CPA(^-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{met} \times \text{asn} (115))</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>(\text{met} \times nd)</td>
<td>1</td>
<td>93</td>
</tr>
<tr>
<td>(\text{met} \times \text{asn} (32.1B))</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>(\text{fol} \times \text{PAB})</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>(\text{fol} \times \text{asn} (32.1A))</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>(\text{pro} (102.\text{B}) \times \text{PAB})</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>(\text{pro} (102.\text{A}) \times nd)</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

| CPA\(^-\) \(\times\) CPA\(^-\)               |                                                   |                                                       |                                                   |                                                       |
| \(\text{trp} (32.1) \times \text{asn} (32.1B)\) | 0                                               | 14                                                    | 86                                              |                                                       |
| \(\text{nd} \times \text{asn} (32.1A)\)     | 1                                               | 94                                                    | 5                                               |                                                       |
| \(\text{PAB} \times \text{trp} (32.1)\)     | 2                                               | 86                                                    | 12                                              |                                                       |
| \(\text{gluB6} \times \text{asn} (32.1A)\)  | 1                                               | 82                                                    | 17                                              |                                                       |
| \(\text{gluB6} \times \text{asn} (32.1B)\)  | 1                                               | 81                                                    | 18                                              |                                                       |
The methods are time-saving since only strains which have responded in the same way to both are retained for further physicochemical analysis. They are efficient, since mutants are obtained with a rather high frequency.

The industrial use of *A. oryzae* strains unable to produce CPA in mixed culture, however, may lead to renewed synthesis of this toxin, either by metabolic exchange through the substrate or by genetic information exchanges through anastomoses between vegetative filaments.

Heterokaryon formation between different *A. oryzae* mutant strains, through vegetative anastomoses or through protoplast fusion, was carried out to investigate this possibility. It led to the following results and considerations. (i) There never was any reciprocal nutritional complementation through the substrate in any of the induced auxotrophies we studied. This fact does not exclude that such a complementation may take place for CPA production.

Neither biological method revealed any CPA production in mixed cultures of two CPA- prototrophic strains.

(ii) Between different genotypes, several pairings gave rise to prototrophic sectors on MM medium, while others did not.

When prototrophic sectors did form, our results show that they were heterokaryotic. They contained a small percentage of heterokaryotic conidia and cells, and a large proportion of both parental types. The results of successive subcloning of thalli issued from prototrophic conidia show that both parental types are associated in these conidia. Constant segregation toward the parental genotypes indicates that the cohabitation of nuclei from different types within the same cell is not well tolerated, and that these nuclei never fuse to form a diploid individual. In *A. oryzae*, however, Ishitani and co-workers (9, 11) have described the spontaneous appearance of diploids at very low frequencies, on the order of $10^{-6}$, but these diploids only rarely give rise to haploid recombinants.

(iii) Through heterokaryon formation we noted the effect of complementation on CPA production as follows. Two CPA+ parents gave rise to CPA+ heterokaryons. This result was expected since the union within the same cytoplasm of two genomes each coding for the production of the toxin can only lead to the synthesis of that toxin.

Pairings between CPA+ and CPA- parents led to CPA+ heterokaryons, except in one case (*pro* [102.A] × *nd*) in which heterokaryons were CPA-. These results can be compared with the observations made on the conidia and hyphal fragments formed by such heterokaryons. Indeed, conidia and hyphal fragments of the CPA+ parent were predominant except in the case of *pro* [102.A] × *nd*, in which the cells of the CPA- parent were more frequent. Thus, the more frequent genotype was expressed.

This disequilibrium in ratios of parental genotypes has also been observed by Malard (Ph.D. thesis) and by Thorbek and Epliv (21). It is probably determined by the level of complementation that induces prototrophy, which might favor one parental genotype over another.

CPA quantification through H-NMR analysis shows that CPA hydroproducer and hyperproducer heterokaryons can be obtained.

The production by heterokaryons of secondary metabolites at rates higher than those produced by their parents can be compared with the observations of Hamlyn and Ball (4) in *Aspergillus chrysogenum*. After protoplast fusion, these authors selected recombinants in this organism which produced higher quantities of cephalosporin C than those produced by the better producing parent.

In the same way, Peberdy and Bradshaw (16) have analyzed the penicillin production of segregants obtained after protoplast fusion between *Aspergillus nidulans*, which produces penicillin, and *Aspergillus rugulosus*, which does not produce this antibiotic. They observed an equal frequency of nonproducer and producer segregants, with several isolates producing penicillin yields higher than those of the *A. nidulans* parent.

Finally, two CPA- parents issued from the same strain or from strains of different origins only produce CPA- heterokaryons. This result implies that all these couples of nonproducers are blocked at the same step in the CPA biosynthesis chain. In such cases, there is no complementation between their genomes united in the same cytoplasm.

Does the coupling of two CPA- individuals in the same culture prevent all risks of producing this toxin? At this stage of our work, it seems that this phenomenon has not been observed, but a firm conclusion may only be reached after pairings or fusions performed with a larger number of different strains are analyzed.

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**LITERATURE CITED**


