An Unstable Strain of Aspergillus foetidus Segregating Proline Auxotrophs

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Two basic colony types have been obtained through single conidial isolation from the Bode strain of Aspergillus foetidus as well as from mutants of this unstable strain. Type I is prototrophic whereas type II is an auxotroph requiring proline. When a type I strain is grown on complex medium it gradually becomes overwhelmed by type II sectors of growth. However, essentially pure cultures of type I can be maintained on minimal medium (lacking proline). The yield of glucoamylase from type II cultures is less than that obtained with type I cultures. The instability of type I cultures when grown on complex medium can not be explained by heterokaryosis or the presence of virus-like particles found in the original Bode strain and its derivatives. The isolation of five stable prototrophic strains obtained as more rapidly growing sectors from type I subcultures grown on complex medium suggests that the instability most probably results from a duplicated chromosomal segment or other chromosomal aberration analogous to those described in A. nidulans.

Genetically unstable strains of filamentous fungi have been accounted for on the basis of spontaneous nuclear gene mutations and selection for the mutant nuclear type in the resultant heterokaryon (11). Spontaneous mutations which lead to decreased product biosynthesis (e.g., antibiotics) have been correlated with an increase in the proportion of spontaneously arising mutant types in the nuclear population (11, 18). Other spontaneous variants in filamentous fungi have been explained on the basis of mutant nonnuclear factors which are suppressive, meaning that they limit or interfere with the replication of the wild-type nonnuclear factor leading to a predominance of the mutant nonnuclear factor (2, 14). Specific spontaneous variants which can not be explained by mutations of either nuclear or nonnuclear factors have been attributed to anomalous chromosome mechanics and (or) aberrations (4, 15, 20).

In this paper, we shall describe a spontaneous proline requirement which arises regularly in a prototrophic strain of Aspergillus foetidus Thom and Raper used for the production of glucoamylase. The appearance of the proline requirement correlates with a drop in glucoamylase yield. Evidence will be presented suggesting that this variant probably arises from the deletion of a duplicated chromosomal segment in a manner equivalent to that observed in Aspergillus nidulans (Eidam) Wint. (3, 21, 24).

MATERIALS AND METHODS

Fungal strains. Aspergillus foetidus strain Bode (ATCC 14916) is an ultraviolet (UV)-induced mutant (U.S. Patent 3,249,514, 1966) from which the other mutants described in this paper were derived. The stock cultures used were carried in soil stored at ~20 C. The Aspergillus awamori Nakazawa strain used was the ATCC 22042 (NRRL 3112) isolate.

Media. The complex medium (CM) used was Sabouraud’s maltose agar (Difco). The minimal medium (MM) was Czapek solution agar (Difco) supplemented, as required, with appropriate amino acids at a concentration of 0.25 mg/ml. For certain experiments minimal N medium was used (29). In specific experiments, 0.3 mg of sodium deoxycholate (Sigma Chemical Co.) per ml was added to the above media to restrict colony size (19).

Induction of mutants. Both N-methyl-N'-nitro-N-nitrosoguanidine and UV light were used as mutagens. The procedure for N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis was basically that described by Adelberg et al. (1) except for the following modification. One milliliter of a washed conidial suspension at a density of 2.0 to 5.0 x 10⁸ conidia/ml was added to 20 ml of trishydroxymethylamino-methane-maleic buffer (0.05 M, pH 6.0) containing 400 μg of N-methyl-N'-nitro-N-nitrosoguanidine per ml. After 1 h of incubation at 30 C, the reaction mixture was diluted 1:1,000 in phosphate buffer (0.1 M, pH 7.0) and plated on CM. The above treatment usually killed between 30 to 50% of the conidia in suspension. The procedure for UV mutagenesis has been described previously (8). For the detection of auxotrophic mutants, conidia from colonies grown on CM were
replicated with sterile velveteen pads onto MM plates and scored for growth or no growth after 18 to 24 h of incubation at 31 to 32°C.

**Cultural conditions for glucoamylase production.** The cultural conditions and medium used for glucoamylase production have been described (8). In addition to the regular corn mash medium, a medium consisting of minimal N salts supplemented with 2% maltose was used for glucoamylase production.

**Glucoamylase [α-D-(1 → 4)-glucan 1,4-glucanohydrolases, E. C. 3.2.1.5] activity assay.** The method used to assay for the glucoamylase activity in the culture filtrate has been described (26). One glucoamylase unit is defined as the activity necessary to form 1 g of glucose from 4 of starch (in a 4% solution) in 0.1 M acetate buffer (pH 4.2) at 60°C in 1 h. The glucose concentration was determined with Schooler's method (7). Preliminary experiments using the glucose oxidase assay (6) have established that glucose was the only detectable reducing sugar released under the above assay conditions.

**Staining of fungal nuclei.** The numbers of nuclei in conidia were determined by staining with azure A following the method described by Pittenger (22).

**Inactivation of conidia by UV.** The kinetics of UV inactivation were studied by preparing a conidial suspension in 0.85% saline from an 8-day-old culture grown on MM or MM supplemented with proline. The resulting suspension was drawn up and down repeatedly in a sterile disposable Pasteur pipet to break up conidial chains. The suspension was pelleted by centrifugation in an International Clinical centrifuge (model CL) and resuspended in fresh saline. The suspension was diluted to 2 to 5 × 10^6 conidia/ml. Ten milliliters of the diluted suspension was poured into a 10-cm petri dish and exposed to UV (dose rate = 14 ergs/mm² per s). The UV system employed has been described previously (16). After various periods of exposure, portions were removed, immediately diluted (where appropriate), and plated on either MM or MM supplemented with proline. All operations were carried out in a room equipped with "gold lamps" (27) to prevent photoreactivation. The plates were incubated at 32°C and the resulting colonies were counted after 96 h.

**Inactivation of conidia by heat.** Conidia were prepared as described for UV inactivation experiments. After centrifugation, the conidia were resuspended in 1.2 ml of saline. One milliliter of washed conidia was diluted into 9 ml of saline which was preheated to 57°C in a constant temperature water bath (Thelec model 84, Precision Scientific Co.). One milliliter of suspension was immediately withdrawn and diluted in 9 ml of saline held on ice to initiate timing of the experiment. Samples were withdrawn from the suspension, diluted in cold saline, or immediately chilled on ice. The chilled suspensions were diluted (where appropriate) and plated on either MM or MM supplemented with proline. Plates were incubated at 32°C for 4 days at which time colony counts were made.

**Preparation of extracts for analysis on rate-zonal sucrose gradients.** Mycelium grown from conidia in liquid minimal N or minimal N supplemented with proline was extracted in a manner identical to that previously described by Banks et al. (5) except that homogenization was accomplished by blending the mycelium for 10 min (in 2.5-min bursts) in a Waring blender. The resulting mixture was sonically treated for 20 min (in 5-min bursts) at 50-W power using a system equivalent to that described previously (28). In addition to examining sucrose density gradient columns for bluish-light scattering zones, specific gradients were monitored with an ISCO model D density gradient fractionator equipped with a model UA-2 ultraviolet (254 nm) analyzer (Instrumentation Specialties Co., Inc.).

**Analysis of sucrose gradient zones by electron microscopy.** All visible light scattering zones or UV-absorbing peak fractions were dialyzed against two changes of cold (4°C) distilled water to remove all sucrose. Portions of the dialyzed material were placed on collodion-coated, carbon stabilized grids which had been exposed to UV irradiation to counteract their hydrophobic tendency (25). After staining with 2% potassium phosphotungstate, pH 7.0, the grids were examined in an RCA EMU-4 electron microscope, equipped with a 35-μm objective aperture and operated at an accelerating voltage of 50 kV.

**RESULTS**

**Properties of original and derived A. foetidus strains.** Strain B42 is a morphological mutant obtained by treating conidia of A. foetidus strain Bode with N-methyl-N'-nitro-N-nitosoguanidine. In contrast to the black conidia produced by strain Bode, the conidial color of the B42 mutant is tan and conidiation is visibly reduced. When tested for glucoamylase yield in corn mash medium, the B42 mutant yields about three times as much enzyme as does the parental strain (15.2 versus 5.2 U/ml). When the fermentation was carried out in minimal N salts supplemented with 2% maltose as the carbon source, the mutant yields about twice as much enzyme as compared to the parental strain (0.98 versus 0.58 U/ml).

When the conidia of the B42 strain were plated on CM, two basic colony types were observed: one displayed heavy sporulation and light-yellowish mycelia (type I) whereas the other produced scattered conidial heads and bright-yellowish mycelia (type II). However, when conidia were plated on MM, only type I colonies appeared after 3 days of incubation. Later experiments showed that conidia from a type II colony would not grow on MM after 3 days of incubation without proline supplementation. When tested for glucoamylase yield, the type II strain produced enzyme at the level characteristic of the original Bode strain (4.9 U/ml in corn mash medium; 0.44 U/ml in
minimal N supplemented with proline and arginine).

The type I phenotype is unstable since it has not been possible by plating single conidia on CM to isolate stable type I strains which do not segregate type II colonies after extended incubation. Type I colonies, of course, can be maintained as an essentially pure line on MM because the type II strain requires proline for growth.

**Effects of subculturing conidia derived from a type I colony on the proportion of type I and II conidia in the conidial population and on glucoamylase yield.** Since the type II strain appears to be at a competitive advantage on CM when compared to type I strains, the proportion of type I conidia in conidial populations derived from a type I colony maintained on MM would be expected to decline if mass conidial transfers are made on CM. Since type I strains are prototrophic, the proportion of type I conidia in conidial populations derived from a type I colony maintained on MM would be expected to remain relatively constant if mass conidial transfers are made on MM. Furthermore, the yield of glucoamylase would be expected to reflect the relative proportion of type I versus type II conidia in a particular conidial population used as inoculum. To test these expectations concerning subculturing a type I strain on CM and MM, conidia produced by a typical type I colony maintained on MM were used to inoculate both media. After incubation, conidial masses from each culture were further transferred on the same medium. At each step of subculturing, conidial suspensions from each culture were plated on CM for scoring of colony types (type II colonies were identified by their morphology as well as failure of replicated conidia to grow on MM) and tested for glucoamylase yield. The proportion of type I colonies in conidial populations after culturing on CM declines exponentially (Fig. 1). The decline in glucoamylase yield might also be interpreted as declining exponentially to the level characteristic of type II strains (3 to 4 U/ml). As expected, subculturing on MM results in a constant proportion of type I conidia and constant glucoamylase yield.

**Fluctuation in the proportion of type II conidia in conidial populations derived from independent colonies growing on complex medium.** If the origin of type II colonies on CM reflected adaptation to a new environment (CM or the proline present in the medium), growing up a series of independent colonies derived from single conidia on CM should result in a constant proportion of type II colonies produced by each culture and a uniform effect on glucoamylase yield from conidia of these cultures (since glucoamylase yield is inversely related to the type II conidial fractions; Fig. 1). Thirty-five type I colonies derived from single conidia were subcultured on CM. The proportion of type II conidial among the conidia produced by each colony together with the yield of glucoamylase from each subculture was determined (Table 1). The results show that the proportion of type II colonies in conidial populations derived from independent colonies varied from 3.0 to 32.0% and glucoamylase yield from 5.5 to 15.2 U/ml. These tests demonstrate that the generation of type II is random with time and excludes adaptation as an explanation for the observed instability.

**Variants in other strains of A. foetidus.** When conidia from the Bode strain were plated...
II conidia kinetics of the survival curves type II colonies derived both of derived between (22-119) black or histidine-requiring VOL. productivity glucoamylase UV sensitivity aIn corn 1. The derived experiments are presented in Fig. 2. Colony no. | % Type II | Glucoamylase act (U/ml)*  
--- | --- | ---  
1 | 3.0 | 14.5  
2 | 5.0 | 15.2  
3 | 5.2 | 15.0  
4 | 5.8 | 14.2  
5 | 6.0 | 13.0  
6 | 6.7 | 13.5  
7 | 7.2 | 13.0  
8 | 8.0 | 12.5  
9 | 8.3 | 11.8  
10 | 8.3 | 12.3  
11 | 9.8 | 11.2  
12 | 10.5 | 12.1  
13 | 11.5 | 12.4  
14 | 12.2 | 11.8  
15 | 13.0 | 11.5  
16 | 13.0 | 12.0  
17 | 14.1 | 11.4  
18 | 14.4 | 11.2  
19 | 15.0 | 11.0  
20 | 16.5 | 11.5  
21 | 17.1 | 11.2  
22 | 17.5 | 11.0  
23 | 18.2 | 10.5  
24 | 19.0 | 10.8  
25 | 19.2 | 9.8  
26 | 20.0 | 9.2  
27 | 20.3 | 9.5  
28 | 21.0 | 8.8  
29 | 21.0 | 9.2  
30 | 22.3 | 8.7  
31 | 23.0 | 8.0  
32 | 26.2 | 8.5  
33 | 28.1 | 7.5  
34 | 30.3 | 6.2  
35 | 32.0 | 5.5  

*In corn mash medium.

on CM, strains equivalent to the type I and II seen in the B42 mutant were detected. The only difference between a particular colony type derived from strain Bode and an equivalent one derived from B42 was in the conidial color (black or strain Bode and tan for B42). When a histidine-requiring auxotrophic mutant (22-119) was selected from among a population of colonies derived from UV-treated conidia of the B42 strain, it too yielded two colony types, both of which required histidine, whereas the type II required both histidine and proline.

**UV sensitivity of type I and II conidia.** The kinetics of inactivation of *A. foetidus* type I and II conidia by UV was investigated. The results of these experiments are presented in Fig. 2. The survival curves obtained were those typical of other fungi exhibiting an initial shoulder and a final exponential slope (16, 27). The survival of type I conidia appears identical whether platings were made on MM or MM supplemented with proline. Conidia from a type II culture appear slightly more sensitive to UV than those derived from a type I culture.

**Heat inactivation of conidia from type I and II strains.** Conidia from a type II strain appear to be inactivated exponentially for the first 15-min exposure at 57 C with some suggestion of an altered exponential slope with treatment beyond 15 min (Fig. 3). As was true for UV, type I conidia are more resistant to heat inactivation than are type II conidia. In a single experiment, we determined, by replica plating the surviving colonies to MM, the fraction of proline-requiring colonies among the survivors of heat-treated type I conidia. In this particular experiment, the fraction of proline-requiring colonies in the population of type I conidia was 0.046. Although the points exhibit considerable scatter due to the small number of surviving colonies able to be scored, there does not appear to be any obvious tendency for the fraction of proline-requiring colonies in the conidial population to change with extended heat treatment. Although the kinetics of inactivation differed, equivalent results were obtained at 70 C.

There is a small, but consistent, difference between types I and II in their sensitivity to inactivation by heating. However, a population of conidia derived from the B42 mutant, which contains both types I and II conidia, when heat...
type II replica plating particles virus-like strains with proline treated
proline-requiring experiments involved running (mean of 2
terial concentrated extracts (5, 23).

We were unable to find the same conclusion concerning UV inactivation.

Analysis of types I and II for content of virus-like particles (VLPs). It has been shown that certain strains of A. foetidus carry not less than three morphologically indistinguishable icosahedral VLPs (40 to 42 nm in diameter) which sediment differently in isopycnic centrifugation (5, 23). For this reason, we prepared extracts from types I and II of A. foetidus to look for VLPs in rate-zonal sucrose density gradients. Preliminary experiments involved running material concentrated by high speed pelleting on 10 to 40% sucrose gradients (SW25.1) and looking for visible bluish-light scattering zones. We were able to find three zones in type I and II gradients at levels corresponding to 21, 23, and 25% sucrose. Each of these zones contains VLPs which are morphologically indistinguishable when examined under the electron microscope. Figure 4 is an electron micrograph of material taken from the 25% sucrose zone of a gradient of a type I extract. Since the VLPs had formed crystalline arrays in this particular preparation, the icosahedral character of the particles is evident. The diameter of the particles is approximately 42 nm and two independent analytical centrifuge runs suggest that the S_{w2} of the particle is about 150S as was reported for similar particles investigated by Banks et al. (5).

Having shown that type I and II strains were qualitatively alike in VLP content, we investigated the extractability of type I versus type II which might influence the recovery of VLPs. The results of three independent extraction experiments are presented in Table 2. From the soluble protein data, it appears that the strains were extracted with approximately equal efficiency. The amount of protein which was recovered from resuspension of the high-speed pellet appears about equal for the two strains. The only difference between the strains is in the greater 260-nm absorbing material observed in the resuspended high-speed pellets obtained from type I.

**Isolation of stable type I strains.** In A. nidulans nonreciprocal translocation can lead to chromosomal duplications. Stains with duplications can produce rapidly growing sectors which presumably would have a balanced genome as the result of loss of all or a part of the duplicated

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Strain</th>
<th>Wet weight mycelium (g)</th>
<th>Dry weight mycelium (g)</th>
<th>Total extractable soluble protein (mg) (17)</th>
<th>Total soluble protein in resuspended high-speed* pellet (mg)</th>
<th>Total OD_{260*} resuspended high-speed pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>46.1</td>
<td>4.6</td>
<td>555</td>
<td>17.4</td>
<td>115</td>
</tr>
<tr>
<td>1</td>
<td>II</td>
<td>44.7</td>
<td>4.9</td>
<td>576</td>
<td>17.5</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>51.7</td>
<td>7.7</td>
<td>418</td>
<td>38.4</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>50.0</td>
<td>8.0</td>
<td>404</td>
<td>34.9</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>21.5</td>
<td>3.0</td>
<td>485</td>
<td>34.5</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>23.2</td>
<td>3.0</td>
<td>418</td>
<td>31.6</td>
<td>54</td>
</tr>
</tbody>
</table>

* Extract was pelleted in a type 30 rotor at 29,000 rpm for 2 h.
* OD_{260*} Optical density at 260 nm.

**Fig. 3.** Thermal inactivation of conidia from type I and type II strains of Aspergillus foetidus. Symbols: ●, Type I (mean of three experiments); ▲, type II (mean of 2 experiments); ☐, fraction of proline-requiring strains in the surviving population when heat-treated type I conidia were plated on MM supplemented with proline (0.25 mg/ml) in a single experiment. Proline-requiring strains were identified by replica plating to MM.

**Fig. 4.** Electron micrograph of representative VLPs observed in sucrose gradient zones of both type I and type II strains of Aspergillus foetidus. The particles measure approximately 42 nm. Bar = 100 nm.
segment (21, 24). Cooke et al. (9) and Nga (21) have reported that when strains carrying chromosome duplications are grown in the presence of certain chemicals (p-fluorophenylalanine, caffeine, trypan blue) more rapidly growing substrains were either induced or selected. Mycelial masses from the Bode and B42 strains as well as an isolate of A. awamori were inoculated onto the center of a petri dish containing CM or CM supplemented with p-fluorophenylalanine (100 μg/ml) or caffeine (20 μg/ml). After incubation at 30°C for 7 to 10 days, sectors which appeared to differ from the parent colony in morphology and growth rate were isolated. Although many of these sectors were identical to each other in morphology and growth rate, the average number of sectors per plate could serve as an indication of genetic instability for a given strain (9). In addition to sectors with improved growth rate and morphology, types with deteriorated morphology, particularly with respect to conidiation, were also observed. The results of such an experiment are summarized in Table 3. Notice that only A. foetidus strains Bode and B42 gave rise to sectors indicating that the sectoring is characteristic of the strain rather than an artifact induced by the CM used.

Assuming the number of sectors showed a Poisson distribution, the differences in the number of sectors observed with and without chemicals present in the medium is not significant (12). Thirty of the sectors were again tested for stability in the same manner as the parental strain. Five sectors no longer produced secondary sectors either spontaneously or in the presence of caffeine. All five stable strains were prototrophic. The five stable strains still harbored VLPs in quantities not significantly different from the parental strain, as shown when mycelia were extracted according to the standard procedure. When tested for glucoamylase yield in shake flasks, three of the five stable strains produced approximately the same or slightly increased level of enzyme, whereas two produced much less than the parental strain (Table 4).

**DISCUSSION**

**Heterokaryosis.** The derivation of type II from type I when colonies are grown on CM might represent the selective overgrowth of a component of a heterokaryon which is balanced on MM. A conidial suspension derived from a type I strain growing on MM should be composed of a mixture of homokaryotic and heterokaryotic conidia. The average nuclear number per conidium for the strains investigated is two (8). Assuming that (i) any conidium which germinates directly to form a type II colony on

### Table 4. Glucoamylase yields for stable prototrophic strains obtained from mutant B42

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium from which isolated</th>
<th>Glucoamylase act* (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B42 (control)</td>
<td>CM</td>
<td>12.5</td>
</tr>
<tr>
<td>s-1</td>
<td>CM</td>
<td>13.6</td>
</tr>
<tr>
<td>s-8</td>
<td>CM</td>
<td>8.2</td>
</tr>
<tr>
<td>s-1c</td>
<td>CM + caffeine</td>
<td>9.2</td>
</tr>
<tr>
<td>s-5c</td>
<td>CM + caffeine</td>
<td>12.2</td>
</tr>
<tr>
<td>s-7c</td>
<td>CM + caffeine</td>
<td>13.8</td>
</tr>
</tbody>
</table>

* Average of results from duplicate flasks.

### Table 3. Sectors obtained from independent plates of medium inoculated with mycelial masses of three Aspergillus strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium*</th>
<th>No. of plates with:</th>
<th>Total no. of plates</th>
<th>Total no. of sectors</th>
<th>Mean no. sectors/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. awamori</td>
<td>CM</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CM + p-fpa</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CM + caffeine</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>A. foetidus Bode</td>
<td>CM</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CM + p-fpa</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CM + caffeine</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>A. foetidus B42</td>
<td>CM</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CM + p-fpa</td>
<td>7</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CM + caffeine</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>20</td>
</tr>
</tbody>
</table>

* The number of sectors observed on CM and CM + caffeine are not significantly different at the 0.05 level (12). p-fpa, p-Fluorophenylalanine.
CM (rather than producing type II sectors from an initial type I colony) is homokaryotic for the factor which controls the type II phenotype and (ii) the distribution of nuclei into the conidia is random, it can be estimated that between 0.10 to 0.39 (\(\sqrt{0.01} \) to \(\sqrt{0.15}\)) of the nuclei in the mycelium must carry the factor which controls type II. This estimate is based on the observation that the fraction of conidia in a suspension prepared from a type I colony growing on MM which germinate directly into type II colonies varies between 0.01 and 0.15. If the instability was based on heterokaryosis, between 0.36 to 0.81 (0.6\(^4\) to 0.9\(^9\)) of the colonies examined in the experiment described in Table 1 should have been homokaryotic for nuclei carrying the factor determining type I. Heterokaryotic colonies should have ranged from 0.18 to 0.47. Since all 35 of the colonies produced type II derivatives, heterokaryosis must be rejected as an explanation for the instability.

Heterokaryosis as a plausible explanation for the observed variability is not yet acceptable on other grounds. Since the parental strain (Bode) from which the B42 mutant was derived also exhibits the variation found in B42, assuming heterokaryosis, the nuclei in the mutagenized heterokaryotic conidium which gave rise to B42 would have had to have mutated in exactly the same way (from nuclei controlling black conidial color to nuclei controlling tan conidial color). This possibility seems even more remote when one considers that the histidine-requiring mutant derived from the B42 mutant exhibited the variation characteristic of the original B42 strain. Again, assuming heterokaryosis, it would have to be assumed that the nuclei in a single heterokaryotic conidium simultaneously mutated to the same requirement, histidine. This seems a highly improbable event.

**Nonnuclear factors.** The possibility was considered that the type II variant might be based on some nonnuclear element perhaps analogous to the petite variant in yeast. The frequency of petite cells may be increased to nearly 100% by growing vegetative cells in a medium containing acriflavine (10). Type I was grown on MM and CM with or without acriflavine at two different concentrations (10 and 100 \(\mu\)g/ml). The yield of type II colonies among the conidia from the colonies grown with or without acriflavine was indistinguishable.

Since VLPs had been demonstrated in other strains of *A. foetidus* (5, 23) as well as other fungi (13), we had to consider whether these particles might in some manner account for the type II variant. We have shown that all the strains derived from the original Bode strain, including the five stable strains, contain VLPs in apparently equivalent quantities. The only differences between extracts of type I and II strains is in the small but consistently greater amount of 260-nm absorbing material in the resuspended high-speed pellet of type I (Table 2). This may reflect a slightly greater quantity of VLPs in type I versus the type II strain. We have repeatedly attempted to isolate a VLP-free strain by UV inactivation (Fig. 2) and heat inactivation (Fig. 3). Among the survivors of such experiments, we have looked for VLP-free strains without success. Since the difference in 260-nm absorbing material between type I and type II strains is minimal (Table 2), we conclude that the VLPs are not related to the genetic instability characteristic of these strains.

**Chromosomal aberrations and genetic instability.** In *A. nidulans*, chromosome duplications resulting either from nonreciprocal translocations (3, 24) or crossing-over in a heterozygous inversion (21) can lead to strains which are unstable vegetatively. The strains with duplicated segments are slow growing and at a selective disadvantage when compared to strains with a standard haploid genome. It has been shown that haploid strains carrying duplications are inhibited by *p*-fluorophenylalanine and that, when such strains are grown in the presence of the amino acid analogue, sectors appear which have lost the duplication thus reducing the genome to the genetically stable haploid (24). The instability observed in these experiments could be explained if it is assumed that the type I strain carries a duplication for a chromosome segment carrying the proline gene. If one segment of the duplication were *pro*\(^+\) and the other *pro*\(^-\), the loss of the *pro*\(^-\) segment would account for the stable *pro*\(^+\) strains isolated when type I was grown on CM irrespective of whether *p*-fluorophenylalanine or caffeine were present. The loss of the *pro*\(^+\) segment of the duplication could account for the appearance of type II (proline-requiring sectors) when type I is grown on CM. The variety of sectors observed in the experiment described in Table 3 could represent substrains which have lost various fractions of the duplicated segment. The fact that the stable type I strains and the type II segregants can be isolated as sectors from a slower growing prototrophic isolate (Bode or B42) is consistent with the hypothesis that these substrains have lost a duplicated segment leading to a balanced genome and an increased growth rate. It is not yet
understood, however, why we were unable to obtain stable type I isolates through single conidial isolation.

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LITERATURE CITED