Intergenic Complementation of Glucoamylase and Citric Acid Production in Two Species of Aspergillus

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All auxotrophs of Aspergillus foetidus and all but two auxotrophs of A. niger which we isolated yield glucoamylase and citric acid, respectively, at levels below that of the prototrophic strain from which they were derived. Results of representative heterokaryon tests suggest that the nucleus was principally responsible for the inheritance of citric acid or glucoamylase production. Most somatic diploid strains of A. foetidus gave rise to higher yields of glucoamylase when compared to their haploid component strains. Both heterokaryons and somatic diploid strains of A. niger synthesized between auxotrophs which were simultaneously reduced in citric acid yields also gave rise to enhanced yields when compared with their haploid components. The yields of a heterokaryon and somatic diploid synthesized between two high producers of citric acid were not higher than those of respective haploid components. We concluded from these results that gene dosage (or ploidy) does not increase the yield of citric acid. The apparent enhancement in yields observed in diploids or heterokaryons synthesized between auxotrophs with reduced yields in both species can be interpreted as resulting from intergenic complementation.

Attempts to correlate gene dosage with the amount of corresponding gene products by manipulating the ploidy of microorganisms have been reported in fungi and yeast. Ikeda et al. (5) examined the yields of kojic acid and protease in various haploid, diploid, and polyploid strains of Aspergillus oryzae but did not reach a definite conclusion regarding the effect of gene dosage on product yields. Macdonald et al. (9) found that the heterozygous diploids of Penicillium chrysogenum, synthesized from a strain producing a wild-type level of penicillin and a high-yielding mutant, produced penicillin titers near that of the wild type. On the other hand, Ciferri et al. (4) made a thorough study of the effect of gene dosage on tryptophan synthetase (TST) activity in Saccharomyces and concluded that TST activity is proportional to the dosage of the active gene on a per cell basis but not on a per milligram of protein basis, in haploid, diploid, and tetraploid strains carrying from one to four active alleles.

In an effort to answer the question of whether formation of heterokaryons and diploids could be used as direct methods for improving fermentation yields in filamentous fungi, we examined the yields of two extracellular products produced by two Aspergillus species, namely, glucoamylase \( \alpha\)-d-(1 → 4)-glucohydrolases, EC 3.2.1.3 and citric acid, in haploid as well as in diploid and heterokaryotic strains. Our results show that there is no direct relation between gene dosage and yields of these two products and that many instances of apparent gene dosage effect we observed may have resulted from intergenic complementation.

MATERIALS AND METHODS

Fungal strains. A. foetidus strain B (ATCC 14916) is an ultraviolet (UV) light-induced mutant obtained by Bode (U.S. Patent 3,249,514, 1966). Three UV-induced morphological mutants (designated as UV-21, UV-22, and B42, respectively), each producing nonblack conidia, were derived from strain B and are the parents of all the auxotrophs used in the study of glucoamylase production. The organism used for production of citric acid was originally isolated from soil and was identified as A. niger according to Raper and Fennell (10). A morphological mutant producing brown conidia (designated as AN-4) was derived from the original isolate and was the parent of various auxotrophs used in this study.

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 concentrations of 0.25 mg/ml.

**Induction of mutants.** Conidial suspension at a density of $0.5 \times 10^9$ to $1.0 \times 10^9$ conidia/ml was placed in a 9.0- x 1.5-cm petri dish and UV irradiated (dose rate = 2.3 ergs mm$^{-2}$ sec$^{-1}$) with constant stirring. For auxotroph isolation, mutagenesis was often followed by filtration enrichment (2). Auxotrophic mutants were detected by replication of colonies grown on CM to MM plates with velvetone pads and scoring for absence of growth after overnight incubation at 31 to 32 C.

**Heterokaryosis and diploid isolation.** Heterokaryons between two auxotrophs were forced by the frontier method on CM (1) and maintained on MM. Diploids were isolated by plating at least $10^4$ conidia per plate from heterokaryons on MM and selecting colonies which produced prototrophic conidia. Diploidy was confirmed by streaking conidia from a colony and by measurement of conidial size (L. T. Chang, C. A. Terry, and R. W. Tuveson, Mycologia, in press). For both strains of *Aspergillus*, the conidia are predominantly binucleate (60-80%) and the ratio of diploid versus haploid conidial diameters is 1.2 to 1.4:1.0. The detailed information concerning the diploid frequency, conidial size, and nuclear number per conidium has been published (Chang et al., in press). The diploid strains of both species are relatively stable even on CM; the spontaneous formation of haploid and diploid segregants was less than $10^{-2}$ per transfer of conidial culture. None of the auxotrophs used in diploid synthesis exhibits a significant frequency of spontaneous reversion ($<10^{-4}$), thus excluding the possibility that revertants could have been mistaken for diploids.

**Cultural conditions for citric acid and glucoamylase productions.** The basal medium used for citric acid production was a 20% dextrose solution which had been decationed by ion exchange chromatography using Dowex-50. To the above basal medium, the following salts were added (milligrams per liter): MgSO$_4$, 1,250; KH$_2$PO$_4$, 250; KCl, 48.4; CaCl$_2$, 68.8; Fe(NO$_3$)$_3$, 12H$_2$O, 0.86; CuSO$_4$, 5H$_2$O, 0.40; and ZnSO$_4$, 7H$_2$O, 0.44. For production of citric acid, one drop of inoculum containing $2 \times 10^8$ to $2 \times 10^9$ washed conidia was inoculated into 25 ml of medium in a 125-ml Erlenmeyer flask. The flasks were incubated at 32 C for 7 days without shaking. Dry weight of the mycelial mat was determined after overnight incubation at 85 C. For testing citric acid production by auxotrophs, the appropriate amino acid at a concentration of 0.25 mg/ml was added to the above medium.

Because only a small fraction ($\approx 10^{-4}$) of the conidia produced by a heterokaryon were heterokaryotic (Chang et al., in press), conidia could not be used as inocula for the propagation of heterokaryons. We used a modification of the method of Macdonald et al. (8) for preparation of heterokaryotic inocula. Conidia from both component strains (at least $10^4$ conidia for each strain) were mixed, diluted to 10 ml with physiological saline (0.86%), and poured into a CM plate. After 24 h of incubation at 32 C, the mycelial felt which developed on the agar surface was removed, washed in saline, and homogenized in 5 ml of saline by using a Waring blender. The resulting suspension was used to inoculate medium that was unsupplemented, to ensure the heterokaryotic growth. The concentrations of citric acid in the culture filtrates were determined by 0.1 N NaOH titration by using phenolphthalein (1% in 95% ethanol) as pH indicator and are expressed as percent (%) equivalent of citric acid. Samples were examined for the presence of acids by ascending chromatography in ether: acetic acid: water (13:3:1) (vol/vol/vol). Acid spots were developed by spraying with bromocresol green (0.4% in 95% ethanol). Samples which produced visible spots corresponding to acids other than citric were discarded.

Bode medium (U.S. Patent 3,249,514, 1966) was used for the production of glucoamylase. A 1-ml amount of conidial suspension ($10^4$ to $10^7$ conidia) was inoculated into 50-ml samples of dilute corn mash in triple-baffled flasks and shaken at 300 rpm (rotary, 1-inch [2.54-cm] stroke) and 32 C for 90 h. The mash contained 25 g of ground corn and 20 g of concentrated corn-starch water per liter. The glucoamylase activity in the culture filtrate was assayed by the method of Smiley et al. (11). One glucoamylase unit is defined as the activity necessary to form 1 g of glucose from 4 g of starch (in a 4% solution) in 0.1 M acetate buffer (pH 4.2) at 60 C in 1 h.

Preliminary experiments using various haploid, diploid, and heterokaryotic strains established that the yields of citric acid and glucoamylase obtained under the conditions described above are not affected by growth rate of any particular strain and that extended incubations would not alter the yields significantly.

**RESULTS**

**Pleiotropic effect of auxotrophic mutations.** All auxotrophic mutants isolated from Bode and B42 strains of *A. foetidus* consistently exhibited a reduced yield of glucoamylase (Table 1). For *A. niger*, the yields of

| Table 1. Glucoamylase production by various prototrophic and auxotrophic strains of *A. foetidus* |
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| | Strains | Origin | Conidial color* | Requirements | Glucoamylase yields (U/ml) |
| --- | --- | --- | --- | --- | --- |
| B | UV-21 | B | Brw | None | 4.9 |
| UV-22 | B | Olv | None | 5.1 |
| 22-42 | UV-21 | Olv | Pro | 1.0 |
| 21-203 | UV-22 | Brw | Arg | 1.7 |
| A-57 | B42 | Fwn | Arg | 5.7 |
| A-223 | B42 | Fwn | His | 3.3 |
| 119 | B42 | Fwn | Nic | 3.1 |

*Abbreviations: +, black; Brw, brown; Olv, olive; Fwn, fawn (tan); and Nic, nicotinate.*
citric acid among various auxotrophs varied considerably, ranging from 30 to 100% of their prototrophic parent (Table 2). Many of these auxotrophic mutations were accompanied by morphological changes.

**Inheritance of genes involved in citric acid or glucoamylase production.** The heterokaryon test (6) was performed to determine if the mutations conferring nutritional requirements and simultaneously affecting the product yields were nuclear or cytoplasmic in origin. For both glucoamylase and citric acid production, a heterokaryon between a higher producer and a low producer was synthesized. Conidia from a heterokaryon were plated on CM. Nutritional requirements and product formation of single-colony isolates were determined (Table 3). In each case, the single-colony isolates obtained after heterokaryosis consist of two distinct populations, identical to the two original haploid strains with regard to nutritional requirements and ability to produce glucoamylase or citric acid.

**Glucoamylase and citric acid yields of diploid strains.** Diploid strains of *A. foetidus* and *A. niger* have been synthesized from various haploid auxotrophs in all possible combinations, except in several instances where heterokaryosis could not be forced. Each of the diploid strains of *A. foetidus* gave higher yields of glucoamylase than its haploid component strains (Table 4). The glucoamylase yield of each somatic diploid approaches the yield of the prototrophic haploid strain from which the auxotrophic mutants were derived (Table 1). Since auxotrophic mutants of *A. foetidus* which do not affect glucoamylase yield have not been isolated, we cannot test critically whether or not

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**Table 2.** Citric acid yields of various auxotrophs of *A. niger* as compared to the prototrophic strain from which they were derived

| Strains | Origin | Conidial color | Requirements | Citric acid yield (%) |
|---------|--------|----------------|--------------|-----------------------|
| AN-4    | AN-1   | Brw            | None         | 11.0                  |
| 230     | AN-4   | Brw            | His          | 4.0                   |
| 232     | AN-4   | Brw            | His          | 4.6                   |
| 234     | AN-4   | Brw            | Met          | 12.8                  |
| 337     | AN-4   | Brw            | Lys          | 5.8                   |
| 407     | AN-4   | Brw            | Cys          | 3.6                   |
| 408     | AN-4   | Brw            | His          | 10.8                  |
| 409     | AN-4   | Brw            | His          | 8.0                   |

* Brw, brown.

**Table 3.** Yield of glucoamylase among single-colony isolates recovered from heterokaryon

| Strains used for heterokaryon | Single-colony isolates after heterokaryosis |
|-------------------------------|--------------------------------------------|
|                               | Phenotypes observed | GA yield (U/ml) | Citric acid yield (%) | GA yield (U/ml) | Citric acid yield (%) |
| Strains | Phenotypes* | GA* yield (U/ml) | Citric acid yield (%) | | |
| 22-42   | Olv Pro     | 1.8              | Olv Pro          | 8 | 2.0 ± 0.4 |
| A-57    | Fwn Arg     | 6.0              | Fwn Arg          | 12 | 5.7 ± 0.7 |
| 407     | Brw Cys     | 3.6              | Brw Cys          | 5 | 2.7 ± 0.8 |
| 234     | Brw Met     | 12.5             | Brw Met          | 8 | 9.5 ± 1.2 |

* See footnote, Table 1, for abbreviations.

GA, Glucoamylase.

**Table 4.** Glucoamylase yields of several diploid strains and their respective component haploid strains of *A. foetidus*

| Strains | Ploidy | Phenotypes                         | Genotypes | Glucoamylase yield (U/ml) |
|---------|--------|------------------------------------|------------|---------------------------|
| 22-42   | N      | Olive, proline requiring           | ols pro    | 1.0                       |
| 21-203  | N      | Brown, arginine requiring          | brw arg    | 1.7                       |
| Dp-1    | 2 N    | Black, prototrophic                | ols + pro  | 4.3                       |
| A-57    | N      | Fawn, arginine requiring           | fun arg    | 5.7                       |
| A-223   | N      | Fawn, histidine requiring          | fun his    | 3.3                       |
| Dp-2    | 2 N    | Fawn, prototrophic                 | fun arg    | 8.7                       |
| 119     | N      | Fawn, nicotinate requiring         | fun nic    | 3.1                       |
| 1112    | N      | Fawn, cysteine requiring           | fun cys    | 3.8                       |
| Dp-3    | 2 N    | Fawn, prototrophic                 | fun nic    | 9.2                       |

* See footnote, Table 1, for abbreviations.

GA, Glucoamylase.
a somatic diploid strain will yield product at a higher level than the component haploids.

Unlike the situation with *A. foetidus* where diploids can only be synthesized between two auxotrophs with reduced product yield, diploids between auxotrophs with essentially normal product yield were obtained with *A. niger* (Table 2). The yields of all diploid strains synthesized, regardless of the yields of their respective haploid component strains, were relatively constant (Table 5), and none of the diploids produced significantly more citric acid than the haploid prototroph (Tables 2 and 5).

Citic acid production by heterokaryons. Citric acid production by forced heterokaryons of *A. niger*, like that of somatic diploids, approached the level of the prototrophic haploid (Table 6). No growth was observed when two auxotrophs were mix-cultured in citric acid medium, thus ruling out cross-feeding as the cause of increased yield. No attempt has been made to examine the glucoamylase yields in forced heterokaryons of *A. foetidus* because of the difficulty in maintaining heterokaryons in the complex medium used for glucoamylase production.

**Stability of diploids and heterokaryons in fermentation media.** In many instances, the stability of various strains in fermentation media was examined by macerating the mycelial mats in a Waring blender and plating suitable dilutions on MM and CM. For citric acid production by *A. niger*, neither spontaneous reversion of haploid auxotrophs nor segregation of diploids and heterokaryons was within detectable range ($<10^{-3}$). In glucoamylase production media, the diploids of *A. foetidus* appeared to be stable, whereas most heterokaryons readily dissociated into haploid components.

**DISCUSSION**

All auxotrophs of *A. foetidus* and all but two auxotrophs of *A. niger* yield product at a level below that of the prototrophic strain from which they were derived (Tables 1 and 2). A similar pleiotropic effect of auxotrophic mutations has been reported in *P. chrysogenum* with respect to penicillin yields (7). The continued association of a nutritional requirement and the capacity to produce product among all conidial isolates recovered from heterokaryons (Table 3) led us to conclude that the nucleus is principally responsible for the inheritance of citric acid or glucoamylase production.

In *A. foetidus*, most diploid strains gave rise to higher yields of glucoamylase compared to their haploid components (Table 4). Because (i) all auxotrophs exhibited lower yields of glucoamylase and (ii) the yields of diploids were not higher than that of the haploid prototroph from which the auxotrophs were derived, the apparent enhancement of yields in somatic diploids can most simply be interpreted as resulting from intergenic complementation.

In *A. niger*, our results concerning the yields of citric acid in heterokaryons are similar to those reported by Ciegler and Raper (3). However, the heterokaryons we studied were forced, whereas those of Ciegler and Raper were not. The enhanced yield of citric acid observed in heterokaryons and somatic diploids, derived from low-yielding auxotrophs, can also be interpreted as resulting from intergenic complementation (Tables 5 and 6). The situation here would be analogous to that in *A. foetidus* somatic diploids. It is possible that the mutation which caused nutritional deficiency could

| Strains | Ploidy | Requirements | Dry weight of mycelial mat (g) | Citric acid yield (%) |
|---------|-------|--------------|-------------------------------|----------------------|
| 408     | N     | His          | 0.920                         | 11.1                 |
| 234     | N     | Met          | 0.983                         | 13.5                 |
| Dp-1    | 2N    | None         | 0.895                         | 12.5                 |
| 407     | N     | Cys          | 0.858                         | 3.1                  |
| 408     | N     | His          | 0.920                         | 11.1                 |
| Dp-2    | 2N    | None         | 0.915                         | 12.3                 |
| 230     | N     | His          | ND*                           | 3.8                  |
| 234     | N     | Met          | ND*                           | 13.5                 |
| Dp-3    | 2N    | None         | ND*                           | 9.9                  |
| 407     | N     | Cys          | 0.858                         | 3.1                  |
| 234     | N     | Met          | 0.983                         | 13.5                 |
| Dp-4    | 2N    | None         | 0.930                         | 11.2                 |
| 407     | N     | Cys          | 0.858                         | 3.1                  |
| 232     | N     | His          | 0.872                         | 4.6                  |
| Dp-6    | 2N    | None         | 0.880                         | 9.8                  |
| 337     | N     | Lys          | 0.853                         | 5.8                  |
| 232     | N     | His          | 0.872                         | 4.6                  |
| Dp-8    | 2N    | None         | 0.890                         | 7.6                  |
| 337     | N     | Lys          | 0.853                         | 5.8                  |
| 407     | N     | Cys          | ND*                           | 3.6                  |
| Dp-10   | 2N    | None         | ND*                           | 11.7                 |
| 230     | N     | His          | ND*                           | 4.0                  |
| 232     | N     | His          | ND*                           | 4.6                  |
| Dp-12   | 2N    | None         | ND*                           | 13.4                 |
| 232     | N     | His          | 0.872                         | 4.6                  |
| 409     | N     | His          | 0.910                         | 8.0                  |
| Dp-18   | 2N    | None         | 0.915                         | 12.5                 |

*ND*, Not done.
also affect, directly or indirectly, a gene(s) responsible for glucoamylase or citric acid production (pleiotropic mutations), as reported for penicillin production by *P. chrysogenum* (7). Intergenic complementation resulting from heterokaryosis and somatic diploid formation between two dissimilar low-producing auxotrophs could correct not only the nutritional deficiencies but also the deficiencies in glucoamylase or citric acid potency in both haploid component strains, thus restoring the yield to the level of haploid prototroph from which they were derived.

If ploidy (gene dosage) with respect to citric acid production were critical, one would have expected that the yield of a diploid strain synthesized between two high producers should exceed that of its respective haploid component strains. The results (Tables 5 and 6) show that the yield of the heterokaryon and somatic diploid is no better than the haploid components alone. Since the high-producing auxotrophs are not deficient in their potency to produce citric acid, heterokaryosis and diploid formation did not result in enhanced yield. It may be concluded that ploidy is not important in determining yields of the two products under study.

The level of TST per yeast cell was found to be proportional to the dose of active TST genes (4). Since TST is an intracellular enzyme, whereas both glucoamylase and citric acid are extracellular gene products, one may question whether gene dosage effects operate only with regard to intracellular gene products. It is also possible that the way we expressed our yields, as units of glucoamylase activity or amount of citric acid per milliliter of culture filtrate, failed to reflect the true potency of a fungal strain, since no consideration was given to the amount of growth relative to the yields. However, we managed to express the yields of glucoamylase and citric acid as units of enzyme activity or amount of citric acid per milligram (dry weight) of mycelia (Tables 5 and 6) or per milligram of soluble protein in a few selected instances. The results expressed in this manner did not alter our conclusion as to the effect of gene dosage (or ploidy) on these two extracellular products.

Another interpretation of our results might be

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**Table 6. Citric acid production by heterokaryons and their respective component strains of *A. niger***

| Strains   | "Ploidy" | Requirements | Dry weight of mycelial mat (g) | Citric acid yield (%) |
|-----------|----------|--------------|-------------------------------|-----------------------|
| 408       | N        | His          | 0.920                         | 11.1                  |
| 234       | N        | Met          | 0.983                         | 13.5                  |
| 408 + 234 | N + N    | None         | 0.910                         | 10.5                  |
| 407       | N        | Cys          | 0.858                         | 3.1                   |
| 408       | N        | His          | 0.920                         | 11.1                  |
| 407 + 408 | N + N    | None         | 0.906                         | 9.5                   |
| 407       | N        | Cys          | 0.858                         | 3.1                   |
| 232       | N        | His          | 0.872                         | 4.6                   |
| 407 + 232 | N + N    | None         | 0.895                         | 8.3                   |
| 337       | N        | Lys          | 0.853                         | 5.8                   |
| 232       | N        | His          | 0.872                         | 4.6                   |
| 337 + 232 | N + N    | None         | 0.905                         | 9.3                   |
| 337       | N        | Lys          | NDa                           | 5.8                   |
| 407       | N        | Cys          | NDa                           | 3.6                   |
| 337 + 407 | N + N    | None         | NDa                           | 7.3                   |
| 230       | N        | His          | NDa                           | 4.0                   |
| 232       | N        | His          | NDa                           | 4.6                   |
| 230 + 232 | N + N    | None         | NDa                           | 10.6                  |
| 232       | N        | His          | NDa                           | 4.6                   |
| 409       | N        | His          | NDa                           | 8.0                   |
| 232 + 409 | N + N    | None         | NDa                           | 8.8                   |

*NDa, Not done.*
that the citric acid yields of the high-producing auxotrophs may already have reached the maximum for this organism. This is unlikely, however, since strains producing higher levels of citric acid than these auxotrophs have been obtained (Chang, unpublished data).

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