the two types of lysozyme, might be due either to the test used being insensitive or to the immunization procedures they employed, which was different from ours (they immunized animals with HEWL instead of DEWL). The use of monoclonal antibodies may resolve this point.
The work reported in the present paper shows that some common epitopes do exist on GEWL and DEWL. The cross-reactivity between the two lysozymes is likely to be due to conformational similarities[28], since only a slight sequence homology has been found on these molecules[24]. The existence of conformational similarities is supported by crystallographic observations showing some common domain structures between g-type and c-type lysozymes, despite the fact that the amino acid sequences are almost entirely different.

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1 Altschuh, D., Al Moudallal, Z., Briand, J. P., and Van Regenmortel, M. H. V., Molec. Immun. *22* (1985) 329–337.
2 Arnheim, N., and Hindenburg, A., J. biol. Chem. *248* (1973) 8036–8042.
3 Arnheim, N., and Steller, R., Archs Biochem. Biophys. *141* (1970) 656–661.
4 Atassi, M. Z., and Lee, C. L., Biochem. J. *171* (1978) 419–427.
5 Benjamin, D. C., Berzofsky, J. A., East, I. J., Gurd, F. R. N., Hannum, C., Leach, S. J., Margoliash, E., Michael, J. G., Miller, A., Prager, E. M., Richelin, M., Sercarz, E. E., Smith-Gill, S. J., Todd, P. E., and Wilson, A. C., A. Rev. Immun. *2* (1984) 51–67.
6 Blake, C. C. F., Mair, G. A., Noth, A. C. T., Phillips, D. C., and Sarma, V. R., Proc. R. Soc. Lond. *B167* (1967) 365–377.
7 Breton, C., Phan Thanh, L., Dubray, G., and Paraf, A., J. Sci. Food Agric. *47* (1989) 311–325.8.
8 Canfield, R. E., Kammerman, S., Sobel, J. M., and Morgan, F. J., Nature, New Biol. *232* (1971) 16–17.
9 Dianioux, A. C., and Jollès, P., Biochim. biophys. Acta *133* (1967) 472–479.
10 Djavadi-Ohaniance, L., Friguet, B., and Golberg, M. E., Biochemistry *23* (1984) 97–104.
11 Engvall, E., and Perlmann, P., J. Immun. *109* (1972) 129–135.
12 Grütter, M. G., Weaver, L. H., and Matthews, B. W., Nature *303* (1983) 828–831.
13 Harper, M., Lema, F., Boulot, G., and Poljak, R. J., Molec. Immun. *24*, no. 2 (1987) 97–108.
14 Hermann, J., Jollès, J., and Jollès, P., Eur. J. Biochem. *24* (1971) 12–17.
15 Ibrahimi, M. I., Eder, J., Prager, E. M., Wilson, A. C., and Arnon, R., Molec. Immun. *17* (1980) 37–46.
16 Ibrahimi, I. M., Prager, E. M., White, T. J., and Wilson, A. C., Biochemistry *13* (1979) 2736–2744.
17 Jollès, J., Ibrahimi, I. M., Prager, E. M., Schoentgen, F., Jollès, P., and Wilson, A. C., Biochemistry *13* (1979) 2744–2752.
18 Jollès, P., and Jollès, J., Molec. cell. Biochem. *63* (1984) 165–189.
19 Jollès, J., Schoentgen, F., Jollès, P., Prager, E. M., and Wilson, A. C., J. molec. Evol. *8* (1976) 59–76.
20 Maron, E., Eshdat, Y., and Sharon, N., Biochim. biophys. Acta *278* (1972) 243–249.
21 Morissey, J. H., Analyt. Biochem. *117* (1981) 307–310.
22 Phillips, D. C., in: Lysozyme. Eds E. F. Osserman et al. Academic Press, New York 1974.
23 Prager, E. M., Wilson, A. C., and Arnheim, N., J. biol. Chem. *249* (1974) 7295–7297.
24 Schoentgen, F., Jollès, J., and Jollès, P., Eur. J. Biochem. *129* (1982) 489–497.
25 Smith-Gill, S. J., Mainhart, C. R., Lavoie, T. B., Rudikoff, S., and Potter, M., J. Immun. *132* (1984) 963–967.
26 Smith-Gill, S. J., Wilson, A. C., Potter, M., Prager, E. M., Feldmann, R. C., and Mainhart, C. R., J. Immun. *128* (1982) 314–322.
27 Ternynck, T., and Avrameas, S., Immunochemistry *6* (1969) 53–66.
28 Vayday, H. C., Dietzler, D. N., and Ladenson, J. H., Hybridoma *4* (1985) 271–276.
29 Van Regenmortel, M. H. V, Immun. Today *10* (1989) 266–272.
30 Varshney, G. C., Mahana, W., Filloux, A. M., Venien, A., and Paraf, A., J. Food Sci. *56* (1991) 224–227.

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## Variation in regulation of aflatoxin biosynthesis among isolates of *Aspergillus flavus*

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*Abstract.* Two new phenotypes of *Aspergillus flavus* which exhibit novel patterns of aflatoxin production have been identified and characterized. In one of the new variants of *A. flavus*, aflatoxin is made in the absence of carbohydrate and concomitantly with growth, without a lag period. A second variant did not produce aflatoxin in the presence or absence of carbohydrate. Chemical mutagenesis of this nonaflatoxigenic strain resulted in mutant strains which produced aflatoxin on carbohydrate-containing media. The aflatoxin production pattern observed in these mutants resembled the typical production scheme, with a lag period through log phase growth.
*Key words.* Aflatoxin; *Aspergillus flavus*; *Aspergillus parasiticus*; secondary metabolism; genetic regulation; nutritional regulation.

The aflatoxins are a group of polyketide secondary metabolites produced by the filamentous fungi *Aspergillus flavus* and *A. parasiticus*. These compounds are toxic and carcinogenic and frequently contaminate food and feed commodities. Previous investigations on the regulation of aflatoxin production have focused on nutritional factors that enhanced or suppressed aflatoxin biosynthesis[1–4]. Media containing certain carbohy-

drates, e.g. maltose (MMS medium) allowed induction of aflatoxin, but media using peptone as the sole carbon source (PMS medium) did not allow this induction [1].

Another frequent observation involving aflatoxin and other secondary metabolites of fungal and bacterial origin is that biosynthesis occurs only after trophophase, the log phase of growth. The so-called idiophase, the period when growth has greatly slowed or stopped, is the phase when developmental and chemical differentiation occurs [2, 3, 5–7]. We report here two new aflatoxin phenotypes among naturally occurring *A. flavus* strains which vary in commencement of aflatoxin production and provide exceptions to the above-stated relations between growth and the onset of secondary metabolism. One of these phenotypes, designated constitutive or *con*, produced aflatoxin before idiophase on either MMS medium or the ordinarily nonpermissive PMS medium. The second new phenotype, designated repressed or *rep*, produced no aflatoxin on MMS medium but could be mutated to produce aflatoxin on MMS. The aflatoxin production pattern of the mutants from the *rep* strain resembled the typical pattern of synthesis found in aflatoxigenic strains, with a lag period before idiophase, and no production on PMS medium.

*Materials and methods*

The sources and phenotypes of the strains used in this study are summarized in the table.

Mutants were induced using N-methyl-N′-nitro-N-nitrosoguanidine (NTG)[8] at concentrations of 0.02% or 0.1%. Strains producing large amounts of aflatoxin could be detected by observing the long wave UV-induced fluorescence of aflatoxin around colonies grown at room temperature. The colonies originated from mutagenized spores plated onto MMS[1] or coconut[9] media containing 0.1% sodium deoxycholate (Sigma). Aflatoxin production was confirmed and quantified as follows. Dry conidia (ca $10^5$), sterilely collected with a wire loop from colonies growing on sporulation medium[10], were inoculated onto the surface of 1 ml liquid MMS medium (supplemented with 1 µg thiamine for strain 46115) in 1-dram screw cap vials (Wheaton Scientific, Millville, New Jersey). The vials were loosely capped, covered, and incubated in the dark at room temperature for 7 days. Aflatoxin determinations on 50-µl samples from these vials were by ELISA[11]. After sampling, 50 µl methanol was added to each sample. Samples were then diluted a minimum of 198 times before using in ELISA. Minimum sample aflatoxin concentration detectible by this method was 2 ng/ml. The ELISA data were logit-transformed[12] and compared to a linear regression analyzed, logit-transformed standard curve on the same ELISA plate. Values represent the mean of three replications. The average coefficient of variation (CV) for the three replications of each aflatoxin-producing strain was 66%. Therefore, the average standard deviation for the three replications of each of these strains was 66% of the mean of those replications. Thin layer chromatography[13] was occasionally performed to validate ELISA data.

Phenotypes of *Aspergillus* strains used in this work

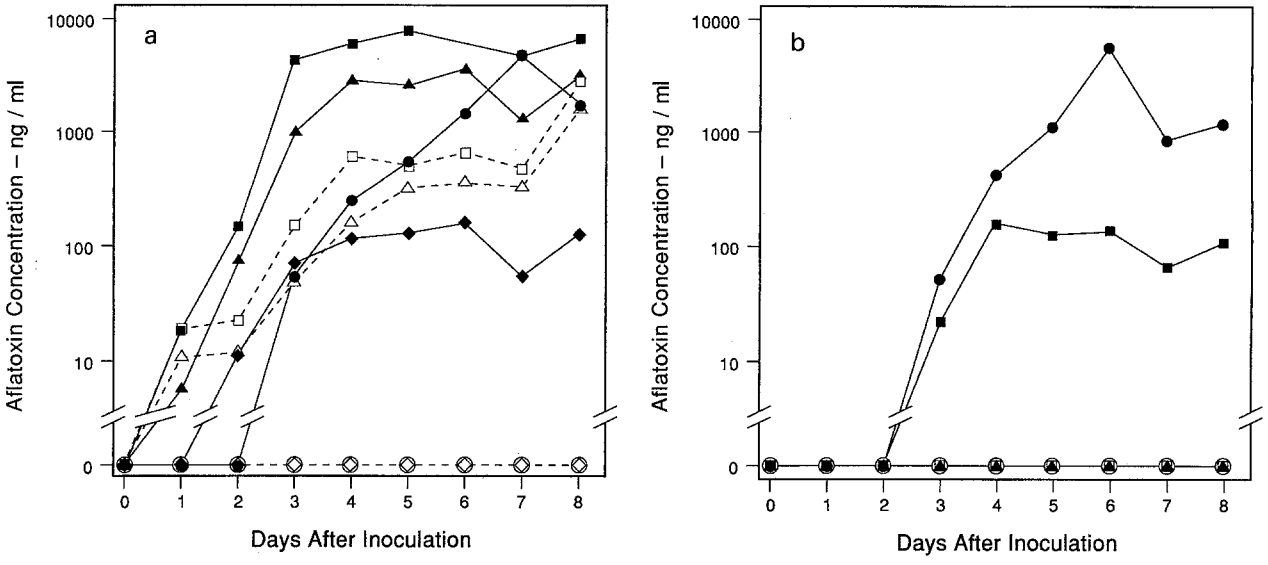
| Strain | Source | Phenotype[a] Aflatoxin concentrations ng/ml | Regulation | Spore color |
|---|---|---|---|---|
| *A. flavus* | | | | |
| 6554 | NRRL[b] | > 10000 | *con* | gr |
| 26 | SRRC[c] | > 10000 | *con* | gr |
| 46115[d] | ATCC[e] | > 1000 | *reg* | w |
| 283 | SRRC | afl- | – | gr |
| 284 | SRRC | afl- | – | gr |
| 285 | SRRC | afl- | – | gr |
| 286 | SRRC | afl- | – | gr |
| 2042 | SRRC | afl- | – | gr |
| 1004 | SRRC | afl- | *rep* | gr |
| brn | Mutant of 1004 | afl- | *rep* | brn |
| afl–3 | Mutant of 1004 | > 10000 | *reg* | or |
| afl–4 | Mutant of 1004br | > 1000 | *reg* | brn |
| *A. parasiticus* | | | | |
| 2999 | NRRL | > 10000 | *reg* | gr |
| 2043 | SRRC | afl- | – | gr |

[a] Phenotype abbreviations: gr, green conidia (wild type), brn, brown conidia; or, orange conidia; w, white conidia, thi, requires thiamine; afl-, no aflatoxin made on MMS; *con*, aflatoxin produced constitutively; *reg*, aflatoxin not produced on PMS medium or during trophophase on MMS medium; *rep*, aflatoxin not produced on MMS but can be mutated to produce aflatoxin on MMS. [b] D. Wicklow, United States Department of Agriculture Northern Regional Research Center, Peoria, IL. [c] M. Klich, United States Department of Agriculture Southern Regional Research Center, New Orleans, LA. [d] K. E. Papa strain 31 a-17. [e] American Type Culture Collection, Rockville, MD.

For time course studies, aflatoxin was determined according to the method described above, except the spores were suspended on the surface of 3-ml media (plus 3 µg thiamine for strain 46115), in 2-dram vials and incubated for eight days. Each day, 50-µl samples were taken from each vial and frozen. Among treatments that contained aflatoxin, the average CV among the three replications for each day was 53%. Growth rates were similar among all strains. Sporulation was never observed before day 3.

*Results*

In tests for aflatoxin formation strains 2999, 46115, 26, and 6554 were grown in PMS and MMS in 3-ml cultures and sampled daily for aflatoxin determination by ELISA over an eight-day period (fig., a). Aflatoxin was detected in all replicate cultures of strains 26 and 6554 within one day of growth on both MMS and PMS media. The other two strains, 2999 and 46115, did not produce aflatoxin on PMS nor within two days of growth (corresponding to trophophase) on MMS, with rare exception (fig., a). There were no apparent differences either in growth rate or in time of initiation of sporulation among these strains. In all cases, sporulation was first detectable on the third day of growth, by which time the




Time course of aflatoxin production by several strains of *Aspergillus*. Dashed lines and open symbols – MPS; solid lines and closed symbols – MMS.
*a* Strains 2999 (circle), 46115 (diamond), 26 (triangle), and 6554 (square). Only one of the three replications of strain 46115 produced aflatoxin on day 2. All other replications of strains 46115 and 2999 started producing aflatoxin on day 3 on MMS. All replications of strains 6554 and 26 on both media produced aflatoxin on day 1.
*b* Strains 1004br (triangle), afl-4 (square), afl-3 (circle).

surface of the medium had been completely colonized. The seven SRRC afl$^-$ strains (table) were tested for aflatoxin production on MMS media with and without the addition of yeast and malt extracts[14]. None of these strains produced aflatoxin on MMS medium. However, when mutagenized conidia of strain 1004 were grown on MMS medium and the medium surrounding the colonies screened for fluorescence under long wave UV light (a characteristic of aflatoxin[13]), mutants surrounded by fluorescent material were detected. Further analysis by ELISA and TLC confirmed that these mutants of strain 1004 produced significant amounts of aflatoxin (table, fig., b). We also found that the wild type parent strain 1004 itself often produced small amounts of aflatoxin (< 100 ng/ml) when grown in MMS medium supplemented with yeast and malt extracts, an indication of the presence in strain 1004 of the entire aflatoxin biosynthetic pathway.

The phenotypes of the three mutants (brn, afl-3, and afl-4) obtained from strain 1004 are summarized in the table. The afl-4 mutant, induced by treatment of the brown conidial mutant of strain 1004 with 0.1% NTG, produced considerably less aflatoxin than the afl-3 mutant and fluorescence from that strain could be seen on coconut agar medium but not on the less sensitive MMS medium.

The 1004 mutants were further examined to characterize their pattern of aflatoxin production. Strains afl-3, afl-4, and the brown conidial mutant of 1004 were grown for eight days on PMS and MMS and sampled daily for aflatoxin analysis (fig., b). The two aflatoxigenic mutants did not make aflatoxin on PMS and exhibited a two-day lag before producing the toxin on MMS, indicating that these strains are of the regulated phenotype previously described for aflatoxigenic aspergilli[1-3].

*Discussion*

The above data indicate the presence of at least three phenotypes varying in expression of aflatoxin production among wild strains of *A. flavus*. The idiophase-restricted phenotype, *reg*, which has been previously described for aflatoxin[1-3] and several antibiotics[2,4,7], is characterized by the strong regulation of secondary metabolite production by nutrient composition of the medium, with production occurring only in late log to stationary phase. This is best exemplified in *A. flavus* by lack of aflatoxin production in the carbohydrate-free medium PMS[1].

The repressed phenotype *rep* (expressed in the wild-type strain 1004), which produced no aflatoxin on MMS medium but could be mutated into aflatoxin production on MMS, has not been previously described for wild strains of aflatoxigenic aspergilli although it may be similar to Papa's afl-1 mutant phenotype, which appeared to contain a dominant suppressor of aflatoxin formation[14]. The presence of the repressed phenotype in natural populations implies that *A. flavus* isolates which do not produce aflatoxin under a particular test condition may have the ability through mutation to produce large amounts of aflatoxin under those conditions. Some strains identified as not being aflatoxigenic in previous studies[15,16] may represent the repressed phenotype, with the potential for causing aflatoxin contamination.

The constitutive phenotype *con* (expressed in strains 26 and 6554) is characterized by aflatoxin production during all stages of growth and development, including during log phase of growth, prior to detectable cell differen-

tiation. Aflatoxin is also made by *con* strains on media lacking carbohydrate as a carbon source. The presence of the constitutive phenotype demonstrates that aflatoxin production need not be tied to cessation of growth. The process of induction of aflatoxin production must therefore be different than induction of sporulation, which is tied to cessation of growth in *Aspergillus*[17].

The differences found among these phenotypes cannot be accounted for by possible variations in the amount of inoculated conidia for each vial (ca $10^5$), since sporulation (indicating idiophase) was always first observed at day 3. This indicates that the variation in the amount of inoculated conidia did not affect initiation of developmental differentiation. The *con* strains always produced aflatoxin within one day, well before idiophase induction, whereas the *reg* strains did not. The qualitative differences among strains of the three phenotypes on MMS and PMS media also cannot be accounted for by potential differences in initial inoculation levels since differences in production occurred throughout the time of growth, long after variation in the initial inoculum was obscured by the large amount of mycelium present.

Although some secondary metabolites are expressed constitutively[18] and others are only expressed in the idiophase, this is the first report which describes both the constitutive and idiophase-restricted phenotypes in different strains of a species that makes a particular secondary metabolite. The presence of these phenotypes provides an opportunity to study the genetic differences between constitutive and idiophase-restricted strains. Such studies may provide genetic models suited for molecular analysis that would explain how nutrition and growth phases regulate formation of aflatoxin and other secondary metabolites.

The presence of these three phenotypes in natural populations implies the presence of a wide variation in aflatoxin regulation. The mutation of a MMS-non-producer to aflatoxin production was effective here in identifying genes which potentially regulate aflatoxin production. Similar experimental designs, as well as parasexual analysis[14] of these mutants may further reveal the genetic nature of this variation.

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1 Abdollahi, A., and Buchanan, R. L., J. Food Sci *46* (1981) 633.
2 Bu'Lock, J. D., in: Regulation of Secondary Metabolite Formation, p. 1. Ed. H. Kleinkauf. Verlag Chemie, 1986.
3 Dutton, M. F., Microbiol. Rev. *52* (1981) 274.
4 Neihaus, W. G. Jr, Exp. Mycol. *13* (1989) 20.
5 Behal, V., TIBS *11* (1986) 88.
6 Demain, A. L., Pure appl. Chem. *58* (1986) 219.
7 Hopwood, D. A., Proc. R. Soc. Lond. B *235* (1988) 121.
8 Papa, K. E., Mycologia *70* (1978) 766.
9 Davis, N. D., Iyer, S. K., and Diener, U. L., Appl. envir. Microbiol. *53* (1987) 1593.
10 Raper, K. B., and Fennell, D. I., in: The Genus *Aspergillus*, p. 41. Williams & Wilkins, 1965.
11 Chu, F. S., Fan, T. S. L., Zhang, G.-S., Xu, Y.-C., Foust, S., and McMahon, P. L., J. Assoc. off. analyt. Chem. *70* (1987) 854.
12 Phelps, D. C., Nemec, S., Baker, R., and Mansell, R., Phytopathology *80* (1990) 298.
13 Stoloff, L., and Scott, P. M., in: Official Methods of Analysis of the Association of Official Analytical Chemists, 14th edn, p. 477. Ed. S. Williams. Association of Official Analytical Chemists, 1984.
14 Papa, K. E., J. gen. Microbiol. *118* (1980) 279.
15 Lillard, H. S., Hanlin, R. T., and Lillard, D. A., Appl. Microbiol. *19* (1970) 128.
16 Koehler, P. E., Hanlin, R. T., and Beraha, L., Appl. Microbiol. *30* (1975) 581.
17 Adams, T. H., and Timberlake, W. E., Proc. natl Acad. Sci. USA *87* (1990) 5405.
18 Yoder, O. C., and Scheffer, R. P., Phytopathology *59* (1969) 1954.

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## A new cell line (XTY) from a tumor of *Xenopus laevis*

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*Abstract.* A new cell line (XTY) was derived from a tumor of a female *Xenopus laevis*. This cell line has been proliferating in standard amphibian culture medium for more than 4 years (470 generations) and has a doubling time of 75.5 h at 25 °C. The cells aggregate into large groups, and their stellate morphology and the expression of desmin demonstrated by immunocytochemistry suggest that their origin is not epithelial.
*Key words. Xenopus laevis*; tumor; cell line; XTY.