IMPROVED MEDIA FOR SELECTING NITRATE-NONUTILIZING MUTANTS IN ASPERGILLUS FLAVUS

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ABSTRACT

Selection and identification of nitrate-nonutilizing (nit) mutants of Aspergillus flavus was compared on six chlorate-containing agar media. Three isolates of A. flavus were used. Nit mutants isolated comprised three phenotypic classes, equivalent to nitaD, nira, and cnx mutants of A. nidulans. There were significant differences among isolates and among media in number and types of nit mutants isolated. The most effective medium contained only nitrate as a nitrogen source, whereas previously described media for isolation of nit mutants contain both nitrate and an amino acid. Morphology of nit mutants was more distinctive on this medium than on other media. The nitrate-only medium yielded mainly nitaD mutants; other media yielded more nira mutants, which complement nitaD mutants. Few cnx mutants were found. Use of a nitrate-only medium in conjunction with another medium should optimize isolation of nit mutants and complementary pairs of mutants.

Key Words: Aspergillus flavus, nitrate-nonutilizing mutants, nita, chlorate, vegetative compatibility

Nitrate-nonutilizing (nit) mutants have been isolated in a variety of organisms (Cove, 1976b). In fungi, nit mutants are useful for testing vegetative compatibility between isolates (Puhalla, 1985; Correll et al., 1987), for studying regulation of nitrogen metabolism (Cove, 1976b), and for providing selectable markers for transformation (Malardier et al., 1989; Unkles et al., 1989; Daboussi et al., 1989). They are particularly valuable in imperfect fungi, in which sexual markers are not available. Unfortunately, nit mutants are difficult to isolate in many fungi, and selective media that work well for certain organisms are useless for others. Our studies of vegetative compatibility in Aspergillus flavus Link, a fungus which is intractable on most chlorate media, led to efforts to develop alternative chlorate media and to compare these with previously described media for efficiency.

Mutations in any one of several genes can produce the nit phenotype. Nit mutants grow very sparsely on Czapek-Dox (CD) agar, which contains nitrate as the sole nitrogen source. Mutants affected at different loci can complement each other when paired on CD agar; the zone of complementation resembles the luxuriant growth of wild-type mycelium. As a rule, complementation occurs between nonallelic nit mutants of a single isolate, and between nonallelic nit mutants of different isolates isogenic at a series of loci governing vegetative compatibility (Puhalla, 1985; Croft, 1987).

Nit mutants are usually isolated on defined media containing nitrate to induce the nitrate reductase system, an amino acid as a nitrogen source for mutant sectors, and chlorate to select for mutants. Although the precise mechanisms are unclear and may differ from fungus to fungus (Correll et al., 1987), cells able to reduce nitrate to nitrite usually reduce chlorate to chlorite, which is toxic (Cove, 1976b). Cells unable to reduce and assimilate nitrate will not be poisoned by chlorite, and will outgrow wild-type hyphae.

Loci have been established for the most common nit phenotypes in A. nidulans (Eidam) Winter (Cove, 1976a, b), and are presumably the same in A. flavus. NitaD mutants lack the structural gene product for nitrate reductase and can utilize nitrite and hypoxanthine; nira mutants lack a regulatory protein needed for synthesis of nitrate and nitrite reductase, and cannot utilize nitrite; and mutants at any of several cnx loci lack a molybdenum-containing cofactor necessary for nitrate reductase and xanthine dehydrogenase and are unable to utilize hypoxanthine.

MATERIALS AND METHODS

All tests were done in parallel on three isolates of A. flavus chosen for diversity in morphology, geographic origin, substrate, and in vitro aflatoxin production. Isolate A came from soil from Yuma, Arizona (#13, Cotty, 1989); isolate B, from a Brazilian peanut (NRRL, A-11608;
Hesseltine et al., 1970); and C, from a walnut (NRRL 3251; Hesseltine et al., 1970). Isolate C was listed by Hesseltine et al. (1970) as an undescribed 'new taxon' because of its abundant, small sclerotia. However, it belonged to A. flavus as defined by Raper and Fennell (1965) and Klich and Pitt (1988), and was indistinguishable from the 'small' isolates of A. flavus described by Cotty (1989).

Six chlorate-containing agar media were compared for ability to select for nit mutants. To ensure that the effect of nitrogen source was not complicated by other factors, all media contained 25 g/L KClO₃ and were adjusted to pH 7.0 with HCI or NaOH prior to addition of 20 g/L Bacto agar. The first medium was CD with chlorate added; this medium contained 3 g/L NaNO₃, as a nitrogen source, and is referred to here as NO3. Four other media contained CD with nitrate replaced by other nitrogen sources: 10 mM glutamate (here called GLU) (Unkles et al., 1989), 10 mM NaNO₃ (NO2), 0.75 mM hypoxanthine (HYX), and 35 mM NaNO₂ with 10 mM L-arginine (NO3+ARG) (Papa, 1986). The sixth medium was Difco potato dextrose agar (PDA) with chlorate (Puhalla, 1985; Correll et al., 1987).

Each isolate was grown on ten Petri plates of each medium. About 10⁴ conidia were seeded into a well in the center of each plate of 25 ml agar. Cultures were incubated in the dark at 30 C and observed weekly. Putative mutant sectors were transferred to CD agar to verify they were nit mutants. Loci responsible for nit phenotypes were determined by growth on CD agar with nitrate replaced by nitrite, ammonium, or hypoxanthine (Cove, 1976a).

Data were analyzed by ANOVA using SAS (Release 6.03, SAS Institute, Inc.). Interactions were noted between the effects of medium and of isolate on number and variety of mutants appearing. Pairwise comparisons were made between means of each isolate on each medium using least squares weighted means (LSMEANS option). Differences between media are presented for each isolate separately.

RESULTS
Differences among media.—Overall, more nit mutants were isolated from NO3 than from the other media (TABLE I). For isolate B, the most mutants were found on NO3+ARG, though NO3+ARG worked poorly for A and C. No mutants were found on PDA.

More NiaD mutants were found on NO3 than on other media for all isolates (TABLE I). NO3 was also the only medium from which niaD mutants were isolated, one each in A and C. All media except PDA yielded more niaD mutants overall than NO3, though the difference was significant only for isolate B.

Colony diameter after 7 days' growth differed significantly between media, and the relative effects of media were the same for all three isolates (TABLE II). Radial growth was negatively correlated with the number of mutants per plate: this correlation was significant for isolates B (r = -0.76, P < 0.05) and C (r = -0.79, P < 0.05), but not for A (r = -0.56, P > 0.10).

Morphology of mutant sectors.—Wild-type growth on chlorate media consisted of sparse, highly branched hyphae with relatively few conidia. Colony margins appeared uneven and scalloped. This morphology is typical of nitrogen starvation (Cove, 1976a). In contrast, wild-type growth on CD was much more luxuriant, with profuse aerial hyphae and conidia, and with more uniform colony margins.

Nit sectors arose differently on different media. On NO3 medium mutants usually appeared at the center of the colony 1-3 wk after inoculation, and gradually outgrew the wild-type mycelia surrounding them. These nit mutants on NO3 were thin, flat, and made few conidia, much like their growth on CD. Hyphae were closely appressed and parallel in orientation, and colony margins were more uniform than those of the wild-type.

In contrast, mutants on NO3+ARG often arose near the periphery of colonies. Most were radial sectors with luxuriant, densely conidiating growth that resembled wild-type growth on CD. Mutants from NO2 looked either like those from NO3+ARG or like those from NO3.

Mutants from HYX and GLU usually arose near the colony periphery as densely mycelial or densely conidiating spots. They usually remained as circular rather than radial sectors, and often the reverse sides of the spots were pigmented. Some sectors from HYX and GLU looked like those described for NO3.

Mutants were most easily recognized on NO3. Some sectors picked off chlorate medium grew like the wild-type when transferred to CD agar.
<table>
<thead>
<tr>
<th>Medium</th>
<th>Isolate A</th>
<th>Isolate B</th>
<th>Isolate C</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nit</td>
<td>niaD</td>
<td>nirA&lt;sup&gt;-&lt;/sup&gt;</td>
<td>nit</td>
</tr>
<tr>
<td>NO3</td>
<td>1.1 a&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.8 a</td>
<td>0.2 ab</td>
<td>0.7 b</td>
</tr>
<tr>
<td>NO2</td>
<td>0.8 ab</td>
<td>0.3 b</td>
<td>0.5 a</td>
<td>0.7 b</td>
</tr>
<tr>
<td>HYX</td>
<td>0.4 bc</td>
<td>0.0 b</td>
<td>0.4 a</td>
<td>0.9 ab</td>
</tr>
<tr>
<td>NO3+ARG</td>
<td>0.0 c</td>
<td>0.0 b</td>
<td>0.0 b</td>
<td>1.2 a</td>
</tr>
<tr>
<td>GLU</td>
<td>0.5 b</td>
<td>0.0 b</td>
<td>0.5 a</td>
<td>0.6 b</td>
</tr>
<tr>
<td>PDA</td>
<td>0.0 c</td>
<td>0.0 b</td>
<td>0.0 b</td>
<td>0.0 c</td>
</tr>
<tr>
<td>5&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Mean number of *nit*, *niaD*, and *nirA<sup>-</sup>* mutants isolated per plate.
* Numbers in a single column not followed by the same letter are significantly different, based on comparisons of least squares weighted means (*P < 0.05*).
† Denotes media on which differences between isolates in number of mutants were significantly different, based on comparisons of least squares weighted means (*P < 0.05*).
### Table II

<table>
<thead>
<tr>
<th>Medium</th>
<th>Isolate</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>( x ) for medium</th>
</tr>
</thead>
<tbody>
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<td>8.5 a</td>
<td>7.9 a</td>
<td>8.3†</td>
<td></td>
</tr>
<tr>
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<td>6.2 b</td>
<td>6.1 b</td>
<td>5.8 b</td>
<td>6.0†</td>
<td></td>
</tr>
<tr>
<td>HYX</td>
<td>6.1 b</td>
<td>6.0 b</td>
<td>5.8 b</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>NO2</td>
<td>4.3 c</td>
<td>4.3 c</td>
<td>5.3 c</td>
<td>4.6†</td>
<td></td>
</tr>
<tr>
<td>NO3+ARG</td>
<td>3.6 d</td>
<td>3.6 d</td>
<td>3.9 d</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>NO3</td>
<td>3.1 e</td>
<td>3.0 e</td>
<td>3.3 e</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>( x )</td>
<td>5.3</td>
<td>5.3</td>
<td>5.3</td>
<td>5.3</td>
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</tr>
</tbody>
</table>

* Mean colony diam at 7 days (cm).
* Numbers in a single column not followed by the same letter are significantly different, based on comparisons of least squares weighted means (P < 0.05).
† Denotes media on which differences between isolates were significantly different, based on comparisons of least squares weighted means (P < 0.05).

These sectors were either not mutants or chlorate-resistant, but not nit mutants (Cove, 1976a; Klittich and Leslie, 1987). When media were ranked according to the percentage of sectors picked that were really nit mutants, the order for each isolate was the same as that for mean number of nit mutants (see Table I). Some mutants had to be transferred to CD agar a second time to remove all wild-type hyphae, but this was equally common for all media.

**Differences among isolates.** Number of mutants obtained differed significantly among isolates on three of the six media, as determined by comparisons of least squares weighted means (Table I). On two of these media, B yielded the most mutants and A yielded the fewest. Number of nitA mutants also differed significantly among isolates on three media; isolate A had the fewest on all three (Table I). Number of nitA mutants differed significantly on only one medium. Colony diameter at 7 days differed significantly on three media, but in each case a different isolate grew fastest (Table II).

**DISCUSSION**

Nit mutants have been used to study vegetative compatibility and genetic diversity within and among populations of *Aspergillus flavus* (Papa, 1986; Bayman and Cotty, 1990), as they have in other fungi. We are also using nit mutants as markers to follow differential survival of isolates co-inoculated into cotton bolls (Cotty et al., 1990). However, their use is often limited by difficulty in isolating mutants; nit media used for *Fusarium* and *Aspergillus nidulans* are not effective for *A. flavus* and many other fungi. Most hymenomycetes, zygomycetes and oomycetes cannot utilize nitrate, so the technique is not applicable to these organisms (Whitaker, 1976).

The mechanism by which nit sectors arise on chlorate media is not understood. It is thought that chlorate is not mutagenic, but rather stimulates and selects for spontaneous mutations (Klittich et al., 1988; Newton, 1988). This distinction is supported by the fact that spontaneous nit mutants of *A. nidulans* tend to be more stable than nitrosoguanidine-induced nit mutants (Newton, 1988). It has been suggested that chlorate acts by stimulating transposon activity, and that differences in sectoring frequency among isolates reflect differences in transposons (Klittich and Leslie, 1988). However, in that case the spontaneous nit mutants should be less stable than those induced by nitrosoguanidine, not more so.

Nit media typically contain both nitrate and another nitrogen source, usually arginine or asparagine. The supplemental nitrogen source presumably feeds the nit sectors, which cannot utilize nitrate. Nitrate presumably induces nitrate reductase activity (Marzluf, 1981), necessary for reduction of chlorate to chloride, and can support wild-type growth until mutation occurs. Our data and those of Unkles et al. (1989) show that a single nitrogen source is sufficient for nit selection; the most successful medium in this study contained nitrate alone. The ability of nit mutants to grow on minimal medium without any known nitrogen source (and to grow on water agar) suggests that enough nitrogen is available in the agar itself or through recycling to support limited growth. Nit mutants were isolated from HYX, NO2, and GLU media, so nitrate was not needed to induce nitrate reductase. Chlorate is known to serve as a substrate for nitrate reductase (Cove, 1976b); it may also be able to induce activity.

The source of nitrogen in the selection medium influenced the ratio of mutant classes, as in *F. moniliforme* Sheld. emend. Snyder & Hansen (Klittich and Leslie, 1988). *F. oxysporum* Schlecht. emend. Snyder & Hansen (Correll et al., 1987), and *A. nidulans* (Cove, 1976a). In *A*
nidulans, glutamate and nitrate medium did not yield nitA− mutants (Cove, 1976a); in A. flavus, nitA− appeared frequently on GLU. In F. moniliforme, hypoxanthine and nitrate gave more nit mutants than nitrate alone or glutamate and nitrate, but yielded fewer nit3 mutants (equivalent to nitA−) than nitrate alone (Klittich and Leslie, 1988), again in contrast to our findings. Ways in which nitrogen sources affect recovery of different mutants are unclear (Cove, 1976a), but the effect of nitrogen source clearly differs from one fungus to another. For each fungus, the optimum medium or combination of media must be determined by experimentation.

Nit mutants of A. flavus grow faster radially than the wild-type on CD (with or without chloride), unlike Fusarium oxysporum (Puhalla, 1985; data not shown). Because nit mutants on NO3 have a distinctive morphology and outgrow the wild-type, we hypothesized that nitA− mutants on NO2 and cnx mutants on HYX might be similarly easy to select. NiaD mutants on these media resemble wild-type growth, since they can utilize hypoxanthine and nitrite. A higher proportion of nitA− was found on NO2 than on NO3 (Table 1). No cnx mutants were found on HYX medium, however.

For complementation studies, two mutants at different loci are more valuable than dozens at the same locus; it, therefore, may be most efficient to use two media in tandem to isolate mutants. Based on the comparisons presented here, chlorate-containing medium with NO3 as a nitrogen source would work well together with hypoxanthine or glutamate medium. Other nitrogen sources such as threonine may be useful for selecting nitA− mutants (Klittich and Leslie, 1988). Cnx mutants may be the most reliable for compatibility tests, because niaD and nitA− mutants from a single isolate do not always complement each other as expected (Correll et al., 1987); however, cnx mutants are rare in A. flavus.

Radial growth can be restricted by increasing chlorate concentration or decreasing pH (Cove, 1976b). Some nit selection media contain as much as 57.6 g/L potassium chlorate (Unkles et al., 1989); this amount will start to crystallize as the agar dries and could probably be reduced several fold if pH was decreased. Preliminary observations show that the NO2 medium discussed here may be more effective with 20 g/L KCIO3, buffered with 0.02 M citrate and adjusted to pH 6.5.

Fungi vary in sensitivity to chlorate and in type and frequency of occurrence of nit sectors, even within species (Cove, 1976a; Klittich et al., 1988). Patterns of inheritance of sectoring frequency in Fusarium moniliforme suggest that chlorate sensitivity is a polygenic trait (Klittich et al., 1988). Differences in number and type of nit mutants among the three isolates used in this study are, therefore, neither surprising nor easily explained. Media discussed here may have to be altered to suit other organisms but should expand the range of fungi in which nit-picking is practicable.

LITERATURE CITED


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