Attempts to Detect an Alternative Vital Role for the Reduced Nicotinamide Adenine Dinucleotide Phosphate-Nitrate Reductase Structural Gene in Aspergillus nidulans

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A possible alternate vital role of the structural gene for reduced nicotinamide adenine dinucleotide phosphate-nitrate reductase in Aspergillus nidulans was tested for and found to be absent in a significant number of haploid and diploid strains.

The reduced nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate reductase (EC 1.6.6.3) of Aspergillus nidulans catalyzes not only the reduction of nitrate by NADPH but also several other reactions, including the reduction of cytochrome c and the oxidation of reduced methyl viologen (6). Mutations in the structural gene for this enzyme, designated niaD, have been described (5, 6). Strains carrying these mutations were detected by their inability to grow on nitrate as a sole nitrogen source, while being able to grow on nitrite, ammonium, urea, or hypoxanthine. Enzyme activity has been measured in over 50 different nia strains, and all have low or nondetectable NADPH-nitrate reductase. In 15 strains, measurements of NADPH-cytochrome c reductase or reduced methyl viologen-nitrate reductase, or both, have also been made, and either or both of these activities have been detected in 12 of these mutant strains (5; D. W. MacDonald, personal communication). Three of these strains did not produce either of these activities but did produce appreciable quantities of NADPH-nitrate reductase cross-reacting material (F. B. Holl, personal communication). The multiple catalytic activity, in addition to the absence of any mutant lacking a detectable niaD gene product, suggested that this product may have some other function in addition to the catalysis of nitrate reduction that is vital for growth on other nitrogen sources. If this were so, the sample of niaD mutants studied would have contained no deletions of the entire niaD locus, as these would have been lethal. This paper describes two experimental approaches designed to test this "vital function" hypothesis. Neither approach provides evidence in its favor.

MATERIALS AND METHODS

A. nidulans strains and genetic techniques. The A. nidulans strains used carried marked genes which are in general use (2, 6). The genetic techniques used were, with minor modifications, those described by Pontecorvo et al. (8). The replica plating technique was that described by Mackintosh and Pritchard (4). Mutant gene symbols used in this paper are as follows: auxotrophies—anA, aneurin; pabaA, p-aminobenzoic acid; pyroA, pyridoxine; and riboB, riboflavin; conidial colors—YA, yellow; fwA, fawn; nitrate reductase structural gene, niaD; nitrate reductase regulatory gene, nir; and cofactor for nitrate reductase activity, cux.

Media and supplements. The standard media and supplements described by Pontecorvo et al. (8) and by Cove (1) were used.

RESULTS

Both experimental approaches, devised to test whether NADPH-nitrate reductase in A. nidulans has an additional vital function, utilized the finding that the majority of mutations which abolish NADPH-nitrate reductase activity also lead to chlorate resistance (7; D. J. Cove, unpublished data). The basis of this phenomenon is thought to be that NADPH-nitrate reductase also catalyzes the reduction of chlorate to some toxic product (probably chlorite). In Aspergillus, nitrate reductase activity can be abolished not only by mutation in the nia gene but also in the cux genes (6) and the nir gene (D. J. Cove, Proc. Roy. Soc. Ser. B. Biol. Sci., in press). Selection for chlorate resistance can yield all these types of mutants, but if resistant strains are selected on medium containing 2.5 mM L-argi-
nine-hydrochloride as sole nitrogen source and 100 mM KClO₃, then almost all such strains are nia mutants (D. J. Cove, unpublished data).

In the first method, a diploid strain was made having a genotype as follows:

\[ \begin{array}{c}
\text{yA1} & \text{pyroA4} & \text{fwA1 niaD17 riboB2} \\
+ & + & + \\
\end{array} \]

Spontaneous chlorate-resistant strains were selected by plating about 10⁶ conidiospores per petri dish of arginine chlorate medium. Resistant strains occurred at a frequency of about 1 per 4 x 10⁷ conidiospores plated. Independent chlorate-resistant strains (150) were isolated, and each was growth tested and shown to have the nia phenotype, i.e., to be unable to utilize nitrate as a nitrogen source. These diploid strains were thus now homozygous at the niaD locus. This homozygosity might have arisen as a result of mitotic recombination between the centromere and the nia locus, which would render the diploid homozygous for the niaD¹ mutation. However, such a recombinational event would also result in homozygosity of the centromere distal riboB2 mutation, which would lead to a requirement for riboflavin. Since selection was carried out in the absence of riboflavin, chlorate-resistant diploids which had arisen in this way would not have been selected.

Whether or not this assumption is valid depends on the mutational lesion in the niaD¹ mutant. If the lesion is a large intragenic deletion or some other mutation that results in no gene product or a grossly altered one, the assumption is not valid. If the lesion is a missense mutation or some other mutation that results in a gene product only slightly different from the wild-type gene product, the assumption may be valid, however, even then the mutation could simultaneously eliminate the nitrate reductase activity and an additional vital function.

Resistant strains could also arise as a result of a mutation in the niaD¹ cistron for nitrate reductase. In this case, since the niaD¹ mutant gene product would be available to provide the additional vital function if it existed, the mutations in the cistron niaD¹ should, within the limits of the experiment, represent the complete spectrum of possible nia mutations, including those leading to the loss of a vital function. Such mutations would, when haploid, be lethal. The 150 different chlorate-resistant diploid strains were haploidized by the method of McCully and Forbes (3) on minimal medium containing 5 mM urea as nitrogen source, 2.4 μM pyridoxine, and 275 μM p-fluoro-DL-β-phenyl alanine. Since the haploidization process affords no opportunity for recombination, the omission of riboflavin from the haploidization medium selects against the fwA1 niaD¹7 riboB2 chromosome. If haploids are recovered, these must carry any or all of the new spontaneous mutations in the nia⁺ cistron which cannot therefore be lethal. Riboflavin-independent haploids were recovered from all the chlorate-resistant diploid strains which were haploidized.

The second method used to test the theory attempted to isolate nia mutations which were temperature sensitive for the vital function. Spontaneous chlorate-resistant strains were selected from two different haploid strains, pabaA1 and anA1 yA1. Each was plated at 10⁶ conidiospores of appropriately supplemented arginine chlorate medium, to which 2 mM sodium deoxycholate had been added to induce tight colonial growth (4), per plate. These plates were incubated at 25 C and gave rise to chlorate-resistant strains at a frequency of about 1 per 3 x 10⁷ conidiospores. Accordingly, 28,200 chlorate-resistant strains, approximately half from each of the two strains, were obtained. A sample of these was shown to consist of over 80% nia mutants. The plates were replicated by using velvet onto appropriately supplemented minimal medium containing ammonium as a nitrogen source. In this way, the resistant strains were tested for an additional temperature-sensitive function essential for growth on ammonium. All of the chlorate-resistant strains were able to grow on the ammonium medium at 37 C.

DISCUSSION

We have been unable to show that NADPH-nitrate reductase has an alternative function vital for growth on nitrogen sources such as ammonium or urea. Although it may have been fortuitous that all of the nia mutants in the initial sample of 15 studied produced a detectable nia product, these results suggest that the majority of these comprise relatively minor alterations. If these are all single-base pair substitutions, they could easily all produce cross-reacting material. We are now looking for a mutant which makes no recognizable nia gene product.

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LITERATURE CITED

phenylalanine with 'master strains' of *Aspergillus nidulans* for assigning genes to linkage groups. Genet. Res. 4:352-359.


