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Cyanide- and Hydroxamate-Resistant Respiration in *Neurospora crassa*

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Strain *inl-89601* of *Neurospora crassa* respires exclusively by means of the mitochondrial cytochrome chain. The respiration of this strain is entirely inhibited by cyanide or antimycin A, the classical inhibitors of cytochrome chain respiration. When this strain was grown in the presence of chloramphenicol, however, two additional terminal oxidases were detected. One of these oxidases is inhibited by substituted hydroxamic acids and has been described previously. The second oxidase was not inhibited by cyanide or hydroxamic acid but was inhibited by azide in the presence of both cyanide and hydroxamic acid. This azide-sensitive respiration was due to a single respiratory pathway with a K_i for azide of 200 μM. A small amount of azide-sensitive respiration was detected in mitochondrial fractions obtained from chloramphenicol-treated cells, and it is likely that the azide-sensitive oxidase is localized in the mitochondrion. The determinants for the azide-sensitive and hydroxamate-sensitive oxidases segregate in a Mendelian manner in crosses and are either unlinked or not closely linked to each other.

Wild-type strains of *Neurospora crassa* respire by means of the standard mitochondrial cytochrome chain that is inhibited by antimycin A or cyanide (8). When the cytochrome chain becomes damaged, due to mutation, inhibition of mitochondrial transcription or translation, or blockade of electron transport, a second respiratory pathway is induced (4-6). This respiration is not inhibited by antimycin A or cyanide but is inhibited by substituted hydroxamic acids (11). Mutants of *Neurospora* have been isolated that cannot produce the hydroxamate-sensitive pathway when grown in the presence of chloramphenicol (2). These mutants have been termed ANT and segregate in a Mendelian manner in crosses. A preliminary report has shown that there are at least two complementation groups of these mutants (2).

We have been attempting to select for new ANT mutants to study the genetics of the hydroxamate-sensitive pathway. The selection procedure used was mutagenesis followed by inositolless death in the presence of antimycin A. We found a new class of mutants that grow poorly in the presence of antimycin A but do not have the hydroxamate-sensitive pathway. These mutants have a novel respiratory pathway that is not inhibited by cyanide, antimycin A, or hydroxamic acids but is inhibited by azide in the presence of both cyanide and hydroxamate.

In this communication we show that this azide-sensitive respiration can be induced in wild-type cells by growth in the presence of chloramphenicol. The determinants for the pathway segregate as nuclear genes and are not closely linked or are unlinked to the determinants for the hydroxamate-sensitive pathway. Our data show that the azide-sensitive pathway is likely to be localized in the mitochondrion. A preliminary report of some of these observations has been presented (D. L. Edwards, *in* H. Degn, D. Lloyd, and G. C. Hill [ed]., *Function of Alternative Oxidases,* in press).

MATERIALS AND METHODS

Strains. Strain *inl-89601* was obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, Calif. Mutant ANT-1 was a gift from J. H. Chalmers and was backcrossed into *inl-89601.* The properties of ANT-1 have been described (2).

Nomenclature. In this communication we use the nomenclature suggested previously (Edwards, in press). The pathways are named by their sensitivity to specific inhibitors: CNS (cyanide sensitive), respiration inhibited by 1.0 mM cyanide, i.e., the cytochrome chain; HAS (hydroxamic acid sensitive), respiration inhibited by 0.78 mM salicylhydroxamic acid (120 μg/ml); AZS (azide sensitive), respiration inhibited by 1.0 mM azide in the presence of the above concentrations of both cyanide and hydroxamic acid.

Respiration measurements. Respiration measurements on whole cells were made as described previously (4). Induction experiments with chloramphenicol were carried out as described (6). The cells were treated with chloramphenicol for 4 h before respiration measurements were made. Mitochondria were isolated as described by Rosenberg et al. (10). Respiration of

isolated mitochondria was determined according to Edwards et al. (6).

Crosses. Crosses were made on corn meal agar after Davis and DeSerres (1). Ascospores were germinated by heat shock at 65°C for 40 min.

Growth tests. Growth tests were carried out in standing tubes of 1.0 ml of Vogel medium N (12) supplemented with 50 μg of inositol per ml and 2% sucrose. Antimycin A was added in 10 μl of ethanol to give a final concentration of 1 μg/ml. Conidia from isolates to be tested were added, and the tubes were incubated at 30°C for 48 h. Growth was determined visually.

RESULTS

Respiration of whole cells. Respiration measurements of cells from strain *inl-89601* are shown in Fig. 1. Trace A shows the respiration of mid-log-phase cells grown in the absence of any inhibitors. The respiration was completely inhibited by 1.0 mM potassium cyanide, indicating that respiration in these cells is due entirely to the mitochondrial cytochrome chain. When these cells were grown in the presence of chloramphenicol, however, other respiratory pathways became apparent. Traces B through F of Fig. 1 show respiration measurements of *inl-89601* grown in the presence of chloramphenicol

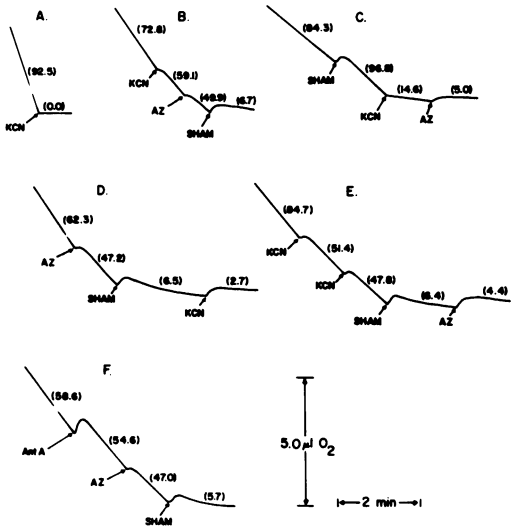

FIG. 1. *Respiration of whole cells of strain inl-89601 after chloramphenicol treatment. Conidia were inoculated at 10^6/ml and grown for 16 h. Solid chloramphenicol was then added to give a final concentration of 2 mg/ml. Measurements were made after 4 h in chloramphenicol. Abbreviations: SHAM, salicyl hydroxamic acid; AZ, sodium azide; Ant A, antimycin A. Final concentrations of inhibitors: KCN, 1.0 mM; SHAM, 0.78 mM (120 μg/ml); AZ, 1.0 mM; Ant A, 1 μg/ml. Ant A was added in 30 μl of absolute ethanol. Numbers in parentheses are respiration rates, in microliters of O_2 per hour per milligram.*

as described in Materials and Methods. The respiration rate in these cells was inhibited by the sequential addition of potassium cyanide, salicylhydroxamic acid, and sodium azide. Each of these inhibitors acted to partially reduce the respiration rate, regardless of the order in which they were added. The presence of all three inhibitors inhibited the total rate of respiration by greater than 90%. The final concentrations of cyanide and salicylhydroxamate used in these experiments was sufficient to completely inhibit respiration due to the cytochrome chain and to the hydroxamate-sensitive pathway (9). These studies indicate the presence in chloramphenicol-treated cells of an additional terminal oxidase that is not inhibited by cyanide or salicylhydroxamic acid but is inhibited by azide. Trace F shows that both azide-sensitive respiration and hydroxamate-sensitive respiration were still present when cytochrome chain respiration was blocked with antimycin A. This is in agreement with previous reports (3, 8) showing that the hydroxamate-sensitive pathway branches from the cytochrome chain before the *b* cytochromes. The location of the azide-sensitive pathway is not known and will be discussed in a subsequent section.

To further demonstrate the presence of the azide-sensitive oxidase in chloramphenicol-treated *inl-89601* cells, a titration experiment was performed. Cells were placed in an oxygen electrode vessel and inhibited with both potassium cyanide and salicylhydroxamic acid. The residual respiration of these cells was then titrated with small portions of sodium azide. A Dixon plot of the data from this experiment is shown in Fig. 2. The plot is linear, indicating a single respiratory pathway, and gives a K_i for azide of 200 μM.

Respiration of isolated mitochondria. We attempted to localize the site of azide-sensitive respiration within the cell and prepared mitochondria from chloramphenicol-treated cells by using the snail gut enzyme procedure described previously (10). We found that a small amount of azide-sensitive respiration segregated with the mitochondrial fraction. Assays of all other fractions produced during the isolation procedure showed no azide-sensitive respiration. Respiration measurements of mitochondria isolated by this procedure are shown in Fig. 3. A small amount of azide-sensitive respiration was seen when reduced nicotinamide adenine dinucleotide was used as a substrate (traces A and B). No azide-sensitive respiration was observed when succinate was used as a substrate (trace C). The rate of azide-sensitive respiration in these mitochondria was small and approached the limit of resolution of our equipment. This

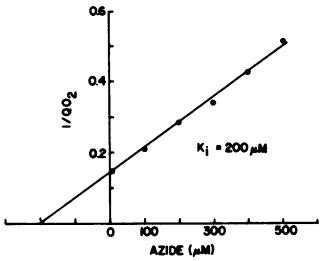

FIG. 2. *Dixon plot of AZS respiration. Chloramphenicol-treated cells were prepared as described in Fig. 1. The cells were poisoned with cyanide (1.0 mM) and salicylhydroxamic acid (0.78 mM), and the residual respiration was titrated by the addition of small portions of azide (30 mM, 10 μl per portion). Corrections have been made for volume changes and a small amount of respiration not inhibited by azide (Fig. 1 and 3).*

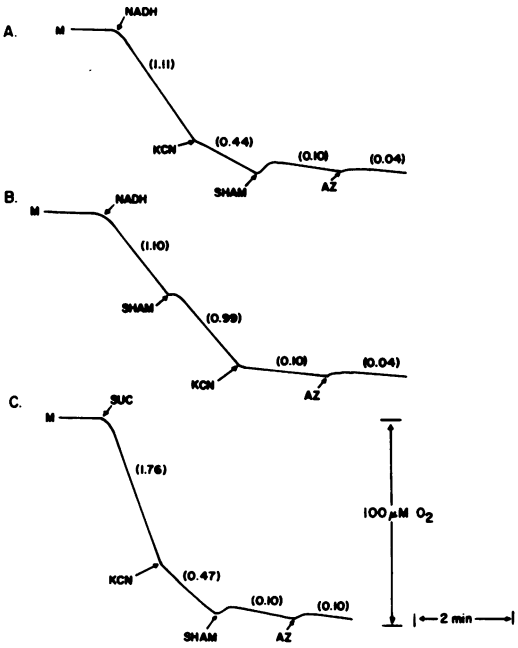

FIG. 3. *Respiration of mitochondria from chloramphenicol-treated cells of inl-89601. Cells were grown as described in Fig. 1. Mitochondria were prepared according to Rosenberg et al. (10). Final concentrations of reagents used: reduced nicotinamide adenine dinucleotide (NADH), 0.5 mM; succinate (SUC), 10 mM; KCN, 1.0 mM; salicylhydroxamic acid (SHAM), 0.78 mM; sodium azide (AZ), 1.0 mM. Mitochondrial content was 0.85 mg in traces A and B and 1.28 mg in trace C. Respiration rates are micromoles per minute per milligram.*

respiration was reproducibly observed in different experiments, however, suggesting that the azide-sensitive pathway is localized within the mitochondrion. The observed rates of reduced nicotinamide adenine dinucleotide oxidation by isolated mitochondria in this experiment were also low but were within reasonable limits, considering the previously reported rate of decay of cytochrome chain respiration by cells grown in the presence of chloramphenicol (6).

Segregation of respiratory phenotypes in a cross. We have previously described a class of mutants that cannot produce hydroxamate-sensitive respiration when grown in the presence of chloramphenicol (2). These have been termed ANT, and their phenotypes segregate as nuclear mutations in a cross. One of these mutants, ANT-1, cannot produce either hydroxamate-sensitive or azide-sensitive respiration when grown in the presence of chloramphenicol. Oxygen electrode traces of chloramphenicol-treated ANT-1 and *inl-89601* are shown in Fig. 4A. ANT-1 was crossed to *inl-89601*, and the respiratory phenotypes of the progeny were determined. Four different respiratory phenotypes were observed in these progeny, two parental and two recombinant types. Examples of each respiratory phenotype obtained are shown in Fig. 4B. A tabulation of the different respiratory

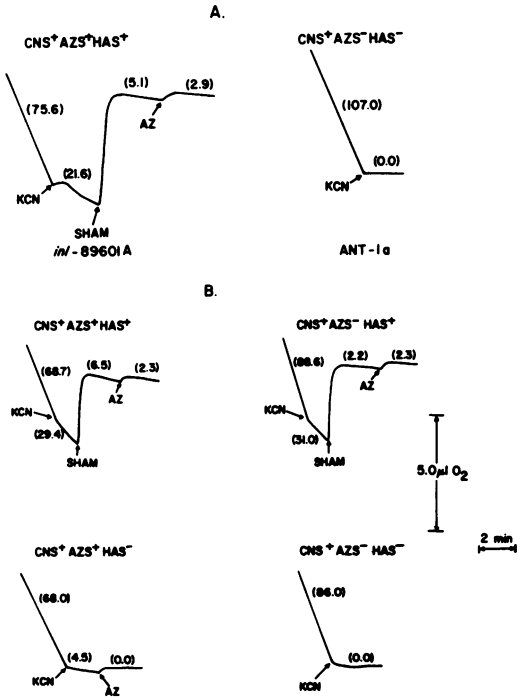

FIG. 4. *Respiratory phenotypes of parental and progeny strains from a cross of inl-89601 A × ANT-1a. (A) Respiratory phenotypes of the parental strains. (B) Representative respiratory phenotypes of progeny from the cross. Cells were grown and treated with chloramphenicol as described in Fig. 1.*

phenotypes in the progeny from this cross is shown in Table 1. The data show that the parental and recombinant phenotypes were present in relatively equal numbers with the exception of the recombinant phenotype $CNS^+/AZS^-/HAS^+$. We have no explanation for this result except that this phenotype is the most difficult to observe experimentally. We cannot exclude the possibility that we simply missed some of the progeny showing this phenotype in our analysis. The data are sufficient, however, to show that the determinants for the AZS and HAS pathways in these strains segregate in a Mendelian manner and are either unlinked or not closely linked.

Growth tests of respiratory phenotypes. We also were able to demonstrate the presence of the azide-sensitive respiratory pathway by means of growth tests in liquid medium containing antimycin A. The procedure used is described in Materials and Methods. When isolates from the cross of ANT-1 to *inl-89601* were analyzed by this method, it was observed that only the double mutants, CNS^+ AZS^- HAS^-, failed to grow in this medium. Mutants of the type CNS^+ AZS^+ HAS^- grew slowly in the presence of antimycin A, whereas mutants of the type CNS^+ AZS^- HAS^+ or CNS^+ AZS^+ HAS^+ grew at a much faster rate. A summary of the relative growth rates of the four respiratory phenotypes found in the progeny is presented in Table 2.

DISCUSSION

The experiments that we have carried out are sufficient to confirm the presence of the AZS oxidase in *Neurospora*. This type of respiration has not been previously reported in *Neurospora* except for a preliminary communication from our laboratory (Edwards, in press). AZS respiration can be demonstrated in chloramphenicol-treated cells regardless of the order of addition of inhibitors (Fig. 1), and titration of the AZS respiration indicates the presence of a single respiratory pathway (Fig. 2). Mutants have been isolated that cannot produce the pathway when challenged with chloramphenicol, and the mutant properties segregate in a Mendelian manner in a cross (Fig. 4). Low levels of azide-sensitive respiration have been observed in isolated mitochondria, and it is likely that the pathway is localized in the mitochondrion (Fig. 3).

TABLE 1. *Respiratory phenotypes of progeny from a cross of inl-89601 to ANT-1*[a]

Phenotype	No.
CNS^+ AZS^+ HAS^+	15
CNS^+ AZS^- HAS^-	12
CNS^+ AZS^+ HAS^-	11
CNS^+ AZS^- HAS^+	4
	42

[a] The cross was carried out on corn meal agar after Davis and DeSerres (1). Germination was greater than 90%. The *inl* and mating-type markers segregated 1:1.

TABLE 2. *Growth properties of respiratory phenotypes in liquid culture*[a]

Phenotype	Growth
CNS^+ AZS^+ HAS^+	++
CNS^+ AZS^- HAS^+	++
CNS^+ AZS^+ HAS^-	+
CNS^+ AZS^- HAS^-	−

[a] Growth tests were carried out in 1.0 ml of Vogel medium N containing 2% sucrose and 1.0 μg of antimycin A. Tubes were incubated at 30°C and scored after 48 h. Symbols: (++) vigorous growth, (+) poor but detectable growth, (−) no detectable growth.

The data in Table 1 show that the gene affecting azide-sensitive respiration in the ANT-1 strain is not closely linked or is unlinked to the gene affecting hydroxamate-sensitive respiration. Azide-sensitive respiration is, therefore, determined independently of those genes coding for the components of the hydroxamate-sensitive pathway, and segregation of these respiratory properties is observed in the progeny from the cross.

Growth tests in liquid medium have also demonstrated the presence of the AZS pathway. The data in Table 2 show that only mutants of the phenotype CNS^+ AZS^- HAS^- fail to grow in the presence of antimycin A, whereas other respiratory phenotypes grow either slowly or rapidly. We have been able to use this method to screen new isolates from various crosses of ANT mutants to wild type. These tests allowed us to segregate the new isolates into three classes: (i) no growth in the presence of antimycin A (CNS^+ AZS^- HAS^-), (ii) poor growth in the presence of antimycin A (CNS^+ AZS^+ HAS^-), and (iii) rapid growth in the presence of antimycin A (CNS^+ AZS^- HAS^+ and CNS^+ AZS^+ HAS^+). We then made respiration measurements on chloramphenicol-treated cultures of the isolates. In over 100 analyses of respiratory phenotypes of isolates from crosses of various ANT mutants to wild type, we have not observed one discrepancy between the growth tests and the respiration measurements. It seems likely to us that the growth tests provide a simple method for obtaining additional HAS^- mutants. We have constructed a strain of the type *inl* CNS^+ AZS^- HAS^+ to be used in further mutation studies. Mutation of this strain to HAS^- should yield cells that fail to grow in the liquid growth tests.

Azide-sensitive respiration of the type described here for *Neurospora* has also been recently reported by Goffeau and his colleagues to

be present in the yeast *Schizosaccharomyces pombe* (7; A. Goffeau, *in* H. Degn, D. Lloyd, and G. C. Hill [ed.], *Function of Alternative Oxidases,* in press). They measured the respiration rate and inhibition constant of azide for this pathway, and their results are remarkably similar to the values that we have obtained.

Although we can demonstrate the presence of the AZS pathway in *Neurospora,* we have no idea of its physiological role. In the laboratory, the pathway can be shown to play an indispensable role by allowing strains that are HAS^- to grow in the presence of antimycin A, which would otherwise be lethal. The physiological role of this pathway, if one exists, must be determined from further studies.

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