COBALT AS A MITOCHONDRIAL DENSITY MARKER IN A STUDY OF CYTOPLASMIC EXCHANGE DURING MATING OF *SCHIZOPHYLLUM COMMUNE*

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ABSTRACT

A slow in vivo uptake of cobalt from a growth medium resulted in an increase in density of mitochondria of *Schizophyllum commune*. Differential labeling of donor and resident mycelia, and subsequent analysis of resident mycelia surrounding donor implants, detected cobalt-dense mitochondria and demonstrated exchange of mitochondria after hyphal fusion. Transfer of mitochondria occurred in fully compatible, common-*A*, and common-*AB* matings, but was not detected in common-*B* matings of the tetrapolar Basidiomycete *S. commune*.

INTRODUCTION

In a tetrapolar organism such as *Schizophyllum commune*, compatibility is determined by two factors termed *A* and *B*. In a fully compatible mating, the *A* and *B* factors of each of the mates differ, e.g., *A1B1* × *A2B2*, and all the events of the normal sexual cycle ensue. These events include hyphal fusion, transfer and migration of nuclei, and establishment of a stable dikaryon (Raper, 1953). The latter is characterized by the presence of two unfused nuclei per cell and by the presence of clamp connections at the septa. During fructification, temporary diploid nuclei are formed in the basidia. Subsequent meiotic divisions result in the production of haploid basidiospores of four different mating types. Thus, the mating types of basidiospores resulting from the mating of homokaryotic mycelia having mating types *A1B1* and *A2B2*, respectively, would be *A1B1*, *A2B2*, *A1B2*, and *A2B1*. In common-*A* matings, in which the mates share in common the *A* factor, i.e., *A1B1* × *A1B2*, nuclear migration also occurs on an extensive scale, but dikaryosis and subsequent events of the sexual cycle do not. In common-*B* matings, i.e., *A1B1* × *A2B1*, defective clamps, designated pseudoclamps, fail to fuse with the adjacent cell. In the common-*AB* mating (*A1B1* × *A1B1*) and in the common-*B* mating (*A1B1* × *A2B1*) nuclear migration is severely limited, and the dikaryotic condition associated with the normal sexual cycle is not established (Raper, 1953; Raper, 1966; Snider and Raper, 1958).

It generally has been assumed that concomitant with the transfer of nuclei is the transfer of cytoplasm. However, no direct test for the transfer of cytoplasm has been available. Some genetic evidence for transfer of cytoplasmic organelles has been obtained with *Neurospora*, *Podospora*, and *Aspergillus* (Diacumakos et al., 1965; Rizet et al., 1958; Jinks, 1959). In the present study, we attempted to modify mitochondrial density with cobalt. Subsequent detection of cobalt-dense mitochondria in unlabeled resident mycelium would thus determine if transfer of cytoplasm occurs

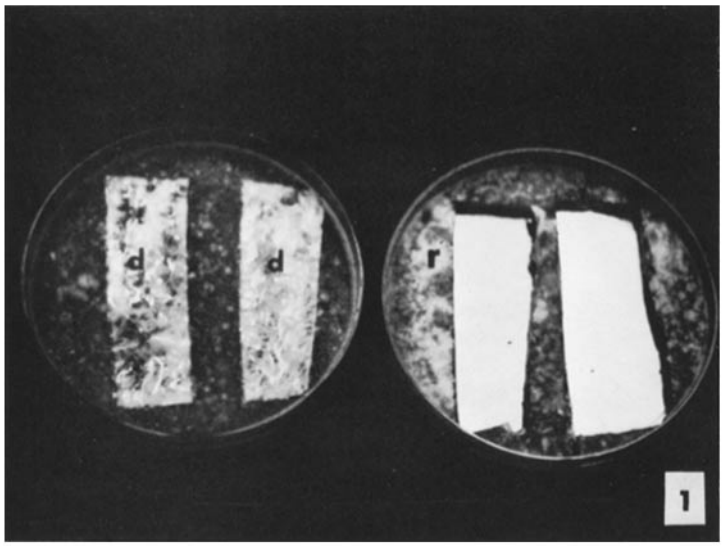

FIGURE 1 Mating of cobalt-labeled donor with unlabeled resident on plate of MC medium. Plate on the left shows the donor implants (*d*) on strips of dialysis membrane placed mcycelial side down onto the unlabeled resident (*r*) mycelium contained on a larger square of dialysis membrane. Plate on the right shows areas of the resident to be extracted for characterization of mitochondria after discarding both the donor implants and the regions of the resident immediately below them.

after hyphal anastomosis. An ability to detect cytoplasmic transfer in the various classes of matings would help to assess its importance in conferring compatibility or incompatibility.

MATERIALS AND METHODS

Strains of *S. commune*, highly isogenic except for mating type (Ellingboe and Raper, 1962), were maintained on 2% agar migration complete (MC) medium (Snider and Raper, 1958), or 3.5% agar MC medium modified by the addition of disodium EDTA (1.56 g/liter), and cobalt chloride hexahydrate (1.0 g/liter). Inoculum was prepared by macerating a 5 cm diameter colony on agar with 50 ml of MC or cobalt broth for 30 s in a Waring blender (Waring Products, New Hartford, Conn.). The hyphal suspension was poured onto sterile squares of dialysis membrane on the corresponding agar medium in Petri plates and incubated 72–96 h at 32°C before use in matings or for extraction and characterization of mitochondria. The cobalt donor mycelium was on agar-free strips of dialysis membrane and applied, mycelial side down, onto MC resident plates prepared as described above. Two donor implants were placed on an MC resident plate for a mating (Fig. 1). After a 24-h incubation at 32°C, donor implants and the areas of the resident immediately below them were removed and discarded. Surrounding resident mycelia on dialysis membranes from a total of six plates were removed for extraction and characterization of mitochondria. The mycelia on the membranes were macerated for 20 s at 4°C in a Waring blender containing 25.0 ml of a solution of 0.25 M sucrose buffered with Tris-HCl (0.01 M, pH 7.0), and 0.001 M EDTA. The homogenate was centrifuged at 4°C for 20 min at 3,000 *g*. The resulting supernate was filtered through 400-mesh nylon before centrifugation at 10,000 *g* for 20 min at 4°C to obtain a crude mitochondrial pellet. The latter was resuspended in 1.0 ml of sucrose buffer as above and applied to a prechilled 0.9–2.0 M linear sucrose gradient prepared according to the methods of Britten and Roberts (1960). The gradients were centrifuged at 4°C at 35,000 rpm (Beckman L2-65B, SW-50L head [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.]) for 4–5 h, to obtain the purified mitochondrial fraction as a distinct band. Fractions were collected from the top of the tube by pumping 2.0 M sucrose, containing neutral red dye, at constant rate through the bottom of the tube. The dye served to indicate the beginning and end of the sample. A continuous absorbancy profile of the effluent from the top of the tube was obtained by monitoring the effluent with a Uvicord II (LKB Instruments, Inc., Rockville. Md.) flow cell at 254 nm. Five-drop fractions were collected and 0.1 ml aliquots of the fractions were tested for succinic dehydrogenase activity. The assay was a modification of a histochemical method (Gomori, 1957), which spectrophotometrically estimated the quantity of the nitro blue tetrazolium (Sigma Chemical Co., St. Louis, Mo., grade III) reduced. The re-

action mixture consisted of 0.1 ml of the fraction being tested and 0.3 ml of a 3:3:3:1 (vol/vol) mixture of succinate (0.25 M), phosphate buffer (0.1 M, pH 7.0), nitro blue tetrazolium (0.1% wt/vol), and sodium cyanide (0.1 M). Incubation was for 1 h at 32°C. Samples were diluted with 1.0 ml of water before determination of absorbancy at 620 nm.

RESULTS

The tolerance of *S. commune* to cobalt complexed with EDTA was determined by measuring the increase in growth of equal size plugs of mycelium transferred from the growing edge of colonies on MC agar medium to liquid MC media containing cobalt and EDTA in various equimolar amounts. At levels up to 0.9 g $CoCl_2 \cdot 6H_2O$ per liter, growth was slightly stimulated. Slight inhibition was apparent at 1.0 g/liter, definite inhibition occurred at 1.5 g/liter, and complete inhibition occurred above 3.0 g/liter.

Effluent from the top of the sucrose gradient tubes containing mitochondria isolated from mycelia grown on MC medium showed maximum absorbance at 254 nm in the region of fraction 8 (Fig. 2). Maximum succinic dehydrogenase activity also occurred at fraction 8 (Fig. 2). On the other hand, maximum absorbance at 254 nm and maximum succinic dehydrogenase activity in tubes containing mitochondria isolated from mycelia which had been grown on the cobalt medium occurred in the region of fraction 10 (Fig. 3). Thus, mitochondria labeled by the uptake of cobalt exhibited a greater density, as indicated by a lower position of sedimentation in sucrose gradients. Experiments in which equal volumes of MC and cobalt-labeled mitochondria were mixed and layered onto gradients indicated that the two populations could be separated by one to two fractions.

The first class of matings to be analyzed for the transfer of cobalt-dense mitochondria was the common-*B*, since preliminary cytological data indicated a lack of transfer of the organelles in that class of matings (Watrud, 1972). Sucrose density gradients of common-*B* matings demonstrated macroscopically the presence of a single mitochondrial band (Fig. 4). Maximum absorbance at 254 nm for these tubes occurred in the region of fraction 8, as did maximum succinic dehydrogenase activity (Fig. 5). These data were interpreted to mean that no transfer of the cobalt-dense mitochondria had occurred; the mitochondria extracted represented those characteristics of the unlabeled resident mycelia only.

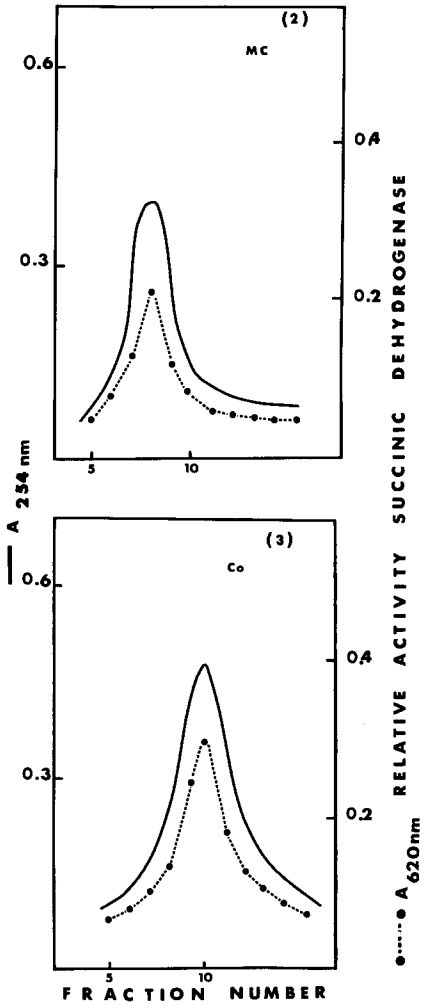

FIGURE 2 Absorbance at 254 nm (——) and succinic dehydrogenase activity (-----) of mitochondria extracted from mycelia grown on MC medium.

FIGURE 3 Absorbance at 254 nm (——) and succinic dehydrogenase activity (-----) of mitochondria extracted from mycelia grown on cobalt medium.

When common-*A* matings were tested, a bimodal distribution of mitochondria was apparent (Fig. 6). Maximum absorbance at 254 nm again occurred in the region of fraction 8, but, in addition, a shoulder was apparent in the region of cobalt-dense mitochondria one to two fractions later. This bimodal distribution was also apparent in the distribution of succinic dehydrogenase activity. This bimodal distribution was interpreted to represent the presence not only of the unlabeled resident mitochondria, but also that of denser

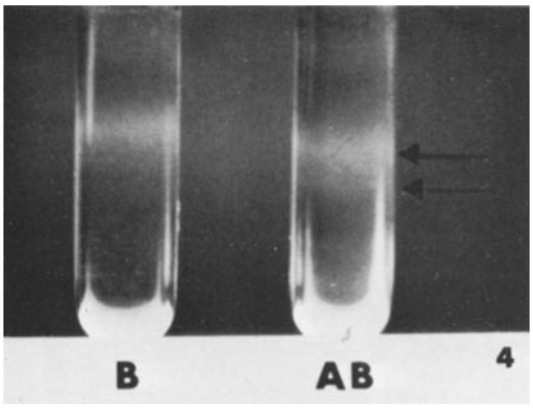

FIGURE 4 Banding patterns of mitochondria extracted from common-*B* and common-*AB* matings and centrifuged in sucrose gradients. Left tube contains mitochondria extracted from the common-*B* ($A \neq B =$) mating, Co *A*41 *B*42 × *A*42 *B*42, and shows single band of unlabeled mitochondria. Right tube contains mitochondria extracted from the common-*AB* ($A = B =$) mating, Co *A*41 *B*41 × *A*41 *B*41, and shows unlabeled resident mitochondria (upper band), and cobalt-dense mitochondria (lower band).

cobalt-labeled mitochondria. The latter are interpreted to have been transferred via hyphal anastomoses in the regions of the donor implants, and to have subsequently migrated beyond the donor implants to the surrounding resident mycelia.

Visual examination of gradient tubes for common-*AB* matings indicated the presence of two mitochondrial bands (Fig. 4). These bands coincided with the observed bimodal distribution of absorbancy at 254 nm, and bimodal distribution of succinic dehydrogenase activity. These maxima occur at fractions 8 and 10 (Fig. 7). Similarly, in Fig. 8, the bimodal pattern is evident in a fully compatible mating, again suggesting a transfer of cobalt-dense mitochondria to nonlabeled residents. The only class of matings studied which did not demonstrate the bimodal pattern was the common-*B*. The specific mating types and cobalt-labeling patterns employed in the various classes of matings are presented in Table I.

To test the possibility of two mitochondrial populations occurring normally in a mating in the absence of cobalt label, unlabeled donors (*A*42 *B*42) were mated with *A*41 *B*41 resident mycelia. Analysis of the mating by sucrose density gradient techniques demonstrated the presence of a single band in the position expected for unlabeled mitochondria.

DISCUSSION

This study represents the first application of induced difference in density of mitochondria to study cytoplasmic exchange shortly after mating, e.g., hyphal anastomosis, in a Basidiomycete. The inheritance of specific populations of naturally differing mitochondrial DNAs in progeny of reciprocal crosses of *Neurospora crassa* and *N. sitophila* has been analyzed by CsCl density gradient techniques (Reich and Luck, 1966), as has that of chloroplast DNAs differentially labeled by ^{14}N and ^{15}N (Sager and Lane, 1972). Differences in density of intact mitochondria isolated from a choline mutant of *N. crassa* exposed in vivo to choline for varying lengths of time, and increases in density of animal mitochondria exposed in vitro to calcium have been detected by sucrose density gradient techniques (Luck, 1965; Lehninger, 1964). Since extended in vivo uptake of cobalt results in a visible darkening of mitochondria in *Saccharomyces* (Lindgren and BeMiller, 1969) and in *Schizophyllum* (Watrud, 1972), it was thought possible that a change in mitochondrial density might also occur.

Analysis by sucrose density gradient techniques as described above indicated that cobalt-labeled mitochondria of *S. commune* differed in density from unlabeled mitochondria. Thus, a means to differentially label donor and resident mycelia was available. The application of the donor mycelia on dialysis membranes free of adhering cobalt agar medium minimized possible contamination of the resident by diffusion of cobalt from a cobalt medium. Discarding areas of the resident which were in contact with the donors as well as discarding the donors, minimized the inclusion of contaminating hyphal fragments from the donors in samples of the residents from which the mitochondria were to be extracted.

Preliminary cytological studies which employed a cobalt vital stain for differential labeling of mitochondria, suggested that mitochondria could be transferred via anastomoses and could subsequently migrate up to 1.2 cm in recipient hyphae (Watrud, 1972). The transfer of mitochondria was detected in fully compatible, common-*A*, and common-*AB* matings, but not in the common-*B* matings. Therefore, if diffusion of cobalt from any cobalt agar medium remaining on the donor mycelia to the resident mitochondria was a factor, we might expect to detect cobalt-dense mitochondria in the resident. We found only unlabeled

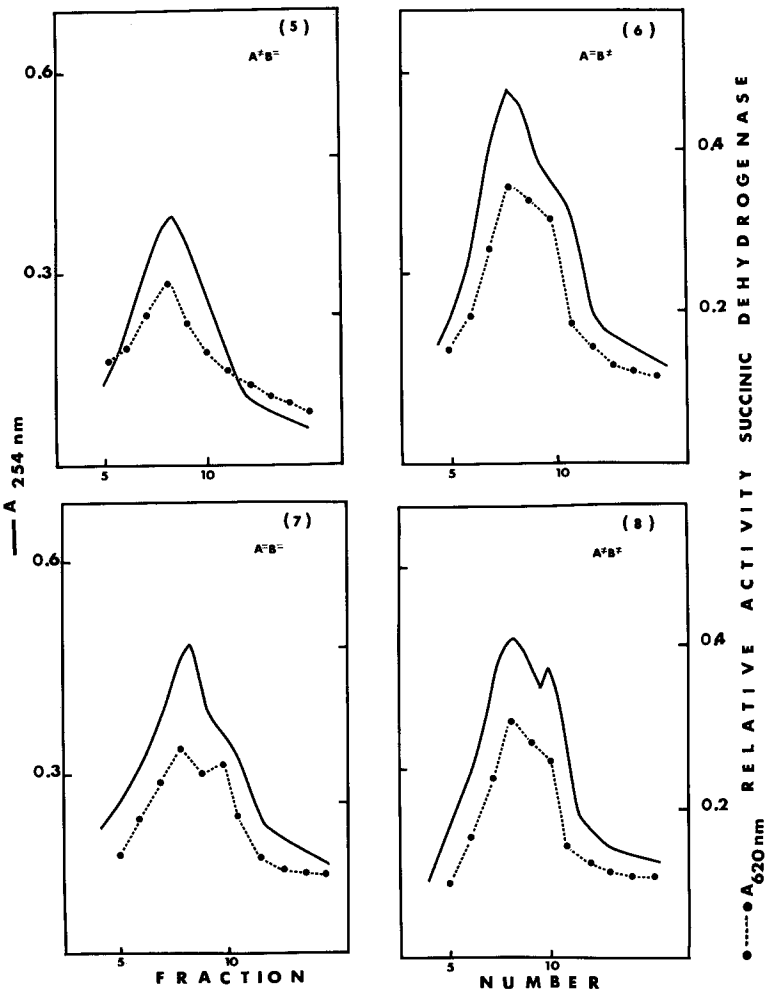

FIGURE 5 Absorbance at 254 nm (——) and succinic dehydrogenase activity (-----) of mitochondria extracted from residents after a common-*B* ($A \neq B =$) mating, Co *A*42 *B*41 × *A*41 *B*41.

FIGURE 6 Absorbance at 254 nm (——) and succinic dehydrogenase activity (-----) of mitochondria extracted from residents after a common-*A* ($A = B \neq$) mating, Co *A*41 *B*42 × *A*41 *B*41.

FIGURE 7 Absorbance at 254 nm (——) and succinic dehydrogenase activity (-----) of mitochondria extracted from residents after a common-*AB* ($A = B =$) mating, Co *A*41 *B*41 × *A*41 *B*41. The presence of unlabeled (resident) and labeled (donor) mitochondria is indicated.

FIGURE 8 Absorbance at 254 nm (——) and succinic dehydrogenase activity (-----) of mitochondria extracted from residents after a fully compatible ($A \neq B \neq$) mating, Co *A*42 *B*42 × *A*41 *B*41. The presence of unlabeled (resident) and labeled (donor) mitochondria is indicated.

mitochondria in resident mycelia after common-*B* matings, as indicated by visual examination, absorbance at 254 nm, and succinic dehydrogenase activity in the gradient tubes. Failure to detect cobalt-dense mitochondria in the common-*B* matings could be due to (*a*) lack of or inhibition of transfer of mitochondria, (*b*) rapid replication of transferred donor mitochondria, with a resultant loss of the density label, or perhaps (*c*) an incompatibility reaction, leading to, e.g., lysis of the donor mitochondria in the resident cytoplasm. That the organelles in question were mitochondria is supported by the coincidence of maxima for succinic dehydrogenase activity, absorbance at

TABLE I

Density Gradient Analysis of Matings

Type of mating	Cobalt donor	MC resident	Replicates	Bimodal pattern of A_{254} nm
Common-*B*	*A*42 *B*41	*A*41 *B*41	2	−
	*A*41 *B*42	*A*42 *B*42	1	−
	*A*42 *B*42	*A*41 *B*42	2	−
Common-*A*	*A*41 *B*42	*A*41 *B*41	4	+
	*A*42 *B*42	*A*42 *B*41	1	+
Common-*AB*	*A*42 *B*42	*A*42 *B*42	1	+
	*A*41 *B*41	*A*41 *B*41	2	+
	*A*42 *B*41	*A*42 *B*41	1	+
Fully compatible	*A*42 *B*42	*A*41 *B*41	4	+
	*A*41 *B*41	*A*42 *B*42	1	+
	*A*42 *B*41	*A*41 *B*42	1	+
	*A*41 *B*42	*A*42 *B*41	1	+

254 nm, and the observed positions of visible bands in the tubes. The use of different sets of strains and/or the alternation of the mate which was labeled with cobalt indicated that the results observed were class specific and not strain specific.

The results reported above were based on observations of 24-h matings. The time interval chosen had to satisfy the following criteria: (*a*) allow sufficient time for the development of anastomoses between the donor and recipient, (*b*) minimize loss of the label due to replication or leaching, and (*c*) minimize the possible accumulation of density label by the resident mitochondria due to diffusion forces. Preliminary analysis of fully compatible and common-*AB* matings at 12, 24, and 48 h by sucrose density gradient techniques as above indicated a unimodal pattern of absorbancy at 12 h, a bimodal one at 24 h, and either unimodal or weakly bimodal ones at 48 h. These were interpreted to mean that at 12 h, anastomoses were not yet formed in sufficient numbers to allow a detectable transfer of cobalt-dense mitochondria, and that by 48 h a significant loss of the density label had occurred, due conceivably to replication or leaching.

With the exception of the common-*AB* matings, the apparent phenomenon of mitochondrial transfer and subsequent migration parallels that of nuclear migration. Nuclear migration is believed to be controlled by the *B* factor (Raper, 1966). The results presented herein suggest that control of mitochondrial transfer and migration may reside in both the *A* and *B* factors. One possible control role for an incompatibility factor on mitochondrial physiology is suggested by data indicating that the level of response to ADP stimulation of respiration in vitro is greater in the wild type than in a strain having a mutant *B* factor (Hoffman and Raper, 1972). A close association of mitochondrial and nuclear membranes (Watrud and Ellingboe, 1973), also suggests possible energy or informational exchange between nuclei and mitochondria. Whether mitochondrial transfer and migration are related to nuclear migration warrants further investigation, as do the mechanisms for movement for each of these organelles.

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