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## Polarity effectors of germ tube outgrowth in conidia of *Neurospora crassa*

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### Abstract

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In the tip of the normally single germ tube outgrowing from conidia of *N. crassa*, frontal mitochondria are ultrastructurally defective and react only faintly to the diaminobenzidine (DAB) reaction for cytochrome oxidase. The ultrastructural defects are generalized to all mitochondria of the swelling conidia treated with sublethal concentrations of the uncoupler (protonophore) 2,4-dinitrophenol ( $10^{-3}$  M 2,4-DNP). Defects are observed in frontal mitochondria of the widened and sometimes double germ tubes outgrown from conidia treated with  $5 \cdot 10^{-4}$  M or stimulated to outgrow normally in  $10^{-5}$  M 2,4-DNP or  $10^{-6}$  M dicoumarol, another uncoupler. The site of outgrowth could be oriented toward a gradient of 2,4-DNP probably acting by local membrane depolarization and mitochondrial uncoupling. The ionophores valinomycin and nigericin can both prevent germ tube outgrowth while showing opposite effects on the membrane potential of mitochondria probed with the fluorescent reagent Rhodamine 123. Elicitation by ethylene glycol of multipolar germ tube outgrowth could be genetically duplicated by the conditionally produced conidia of the morphological mutant "amyccelial". Comparatively to the subapical mitochondria of wild type single germ tubes, those of the 4–6 mutant germ tubes fluoresced only faintly in Rhodamine 123.

Our previous model of apical vesicular traffic self-entrained by a "proton motor" is updated to take into account the consequences of the ultrastructural defects and ensuing respiratory and coupling deficiencies of the frontal mitochondria for the ATP-regulated proton circuitry (tip pumps out, leaks on) driving tip growth.

### Introduction

Phenotypically-induced *multipolar* outgrowth can be produced in the genetically-determined, monopolar germination of macroconidia of *Neurospora crassa* in the presence of ethylene glycol (Bates and Wilson 1973). *Bipolar* germination could be produced by a classical uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (Turian 1980) or by treatment of germinating conidia with anticalmodulin agents (Ortega Perez

and Turian 1986). Such effects suggested interference with polarity at both the levels of mitochondrial activity and calcium gradients.

Polarity of outgrowth from spherical-sub spherical structures, from fucoid eggs to fungal spores, is fixed – and predicted – by the entrance of positive electrical currents (Jaffe 1979, Nuccitelli 1983, Harold 1986) which form side loops from their subapical emission sites. Such currents are sustained by protons extruded subapically by plasmalemmal  $H^+$  ATPase or proton translocase (Slayman 1977). Such processes therefore necessarily elicit a requirement for ATP expected to be produced by mitochondrial energetic activity. Mitochondria being the main producers of such energizing ATP, they would be the source of the tip acidifiers (protons) influxed there as support of such positive electric currents. Mitochondria have also been involved in the primary process of intraconidial polarization through their  $H^+$  ATPase activated by 2,4-dinitrophenol (2,4-DNP) parallel to a speeded up outgrowth of germ tubes in *Neurospora crassa* (Turian and Michéa-Hamzehpour 1983). This effect was ascribed to an ATPase-mediated vectorial extrusion of protons toward a thereby depolarized plasmalemmal zone elected as the outgrowth site.

It was therefore of interest to investigate further both the relevance of mitochondria to the determination of the outgrowth site of the germ tube from conidia of *Neurospora crassa* and the possibility to get a multipolar mutant to obtain further information about the influence of genetic information on polarity through its cytoskeletal control.

## Material and methods

*Neurospora crassa* wild type strain STA<sub>4</sub> (262 A) and the morphological mutant “Amycelial” (Amyc 305 A) have been grown at 25 °C and maintained on synthetic Vogel minimum medium (Vogel 1956). Permissive medium to get macroconidia of “Amyc” was the same synthetic medium but with Na-acetate (2%) + Na-succinate ( $10^{-2}$  M) as C sources. For germination studies, conidia were collected with sterile water, filtered through gauze and centrifuged 5 min at 5000 × g. Erlenmeyer flasks containing 30 ml of medium were inoculated with  $1.2 \times 10^5$  conidia.

For electron microscopy conidia were collected by centrifugation, fixed in glutaraldehyde (2.5%)-OsO<sub>4</sub> (2%), post fixed and stained in uranyl acetate (2%) and lead citrate. Grids were observed in a Zeiss M 10 electron microscope.

The cytochemical test for cytochrome oxidase was performed with the 3,3'-diaminobenzidine (DAB) reaction according to Seligman et al. (1968) and Ton-That et al. (1983). Rhodamine 123 was used as fluorescent probe for evaluation of the membrane potential of mitochondria according to Johnson et al. (1981). The stain was applied for 30 min and the germinative conidia were then washed and finally observed and photographed on a Leitz (Orthoplan-Orthomat) fluorescence microscope.

The inhibitors 2,4-dinitrophenol and dicoumarol were obtained from Fluka; valinomycin and nigericin from Sigma.

## Results and discussion

### I. Phenotypical effects

#### a) Ultrastructure and functional features

On the zygotes of fucoid algae, as well as on conidia and other fungal spores, which have *no* predetermined axis of polarity, the site and direction of outgrowth were suggested to be localized by a cation ( $Ca^{2+}$  or  $H^+$ ) current (Jaffe et al. 1974, Harold 1986).

Its loops around the cell would depend on segregation of pumps and leaks in separate location (Jaffe 1979, Harold 1982). The problem then is to know what factors control the congregation of the leaks at the presumptive outgrowth site of the germ tube in which positive charges enter. Patching factors such as F-actin cables or caps, as recently suggested by McKerracher and Heath (1987), could be internal guiding factors for the selective congregation of the leaks in the apical plasmalemma. However, as pumps are homogeneously distributed and active for  $H^+$ -efflux during the isometric growth stage, they could simply be locally inactivated by polar deficiency in ATP as a result of local uncoupling of mitochondria.

In the normal conditions of germination, few mitochondria are frontally oriented, probably due to some type of genetically-controlled positioning by microtubules (Rossier et al. 1987), and few mitochondria contact the plasmalemma through electron opaque patches which might be uncoupling lipids (oleates, etc.; see Erecinska and Wilson 1981) transiently and locally depolarizing the plasmalemma (Turian and Geissler 1984). Such an hypothesis was proposed by the observation of the stimulated rate of germ tube outgrowth from conidia in the presence of low concentrations of the classical uncoupler 2,4-dinitrophenol (2,4-DNP). This protonophore is known as an active electrical depolarizing agent (Pall 1977). At high concentrations, it would depolarize the whole peripheral plasma membrane and uniformly penetrate into the yellowish conidial cytoplasm. At a sublethal concentration of  $10^{-3}$  M, the conidium can only grow isometrically; its mitochondria are uniformly distributed but present ultrastructural defects such as underdeveloped cristae (Fig. 2a). In bromocresol green ( $10^{-4}$  M), such swollen conidia stain uniformly greenish-yellow (pH 5) thereby revealing their cytoplasmic acidification as previously shown in the heat-treated and also swollen ones (Michea-Hamzehpour and Turian 1984). At the lower concentration of  $10^{-4}$  M, 2,4-DNP allows the outgrowth of only widened germ tube(s) (Fig. 1a); these further grow into hyperbranched colonial mycelia (Crooken and Tatum 1968, Turian 1979), an effect also observed in the Oomycete *Achlya* (Harold and Harold 1986).

Tip acidification as probed with bromocresol green or alizarin yellow S is less noticeable in the shortened and widened hyphae than in the tips of untreated, rapidly elongating hyphae. At the low concentrations of  $10^{-5}$ – $10^{-6}$  M, 2,4-DNP was found to stimulate germination (Turian 1980) as also found with carbonylcyanide p-trifluoromethoxyphenyl-hydrazone (FCCP). Such stimulatory effect has now been extended to another efficient uncoupler, dicoumarol at  $10^{-6}$  M (Fig. 1c).

The stimulatory and site-specifying effect (Fig. 1b) of uncouplers or protonophores may be viewed as a two-step process: exogenous, local depolarization of the plasma membrane, temporally and spatially followed by deenergization of the permeabilized frontal mitochondria dissipating their membranar gradient of protons and leading to a restricted or abolished synthesis of ATP. There would therefore be localized, reduced availability of ATP for the  $H^+$  translocase which electrogenically extrudes protons through the subapical cytoplasm (Slayman 1977, Goffeau and Slayman 1981). Such a deficiency in ATP, and therefore restriction in the outward pumping of protons, could permit  $H^+$  leaks to function freely at the outgrowth site.

We expected that the uncoupling effect of 2,4-DNP could be perceived at the ultrastructural level. This has been confirmed with the restricted internal pattern (diffuse membranes, reduced cristation) observed in all the mitochondria of conidia incubated for germination (stopped or severely delayed) in the presence of the sublethal concentration of  $10^{-3}$  M 2,4-DNP (Fig. 2a). Interestingly, this disruptive effect remains limited to frontal mitochondria in the germ tube outgrown from conidia stimulated to germi-

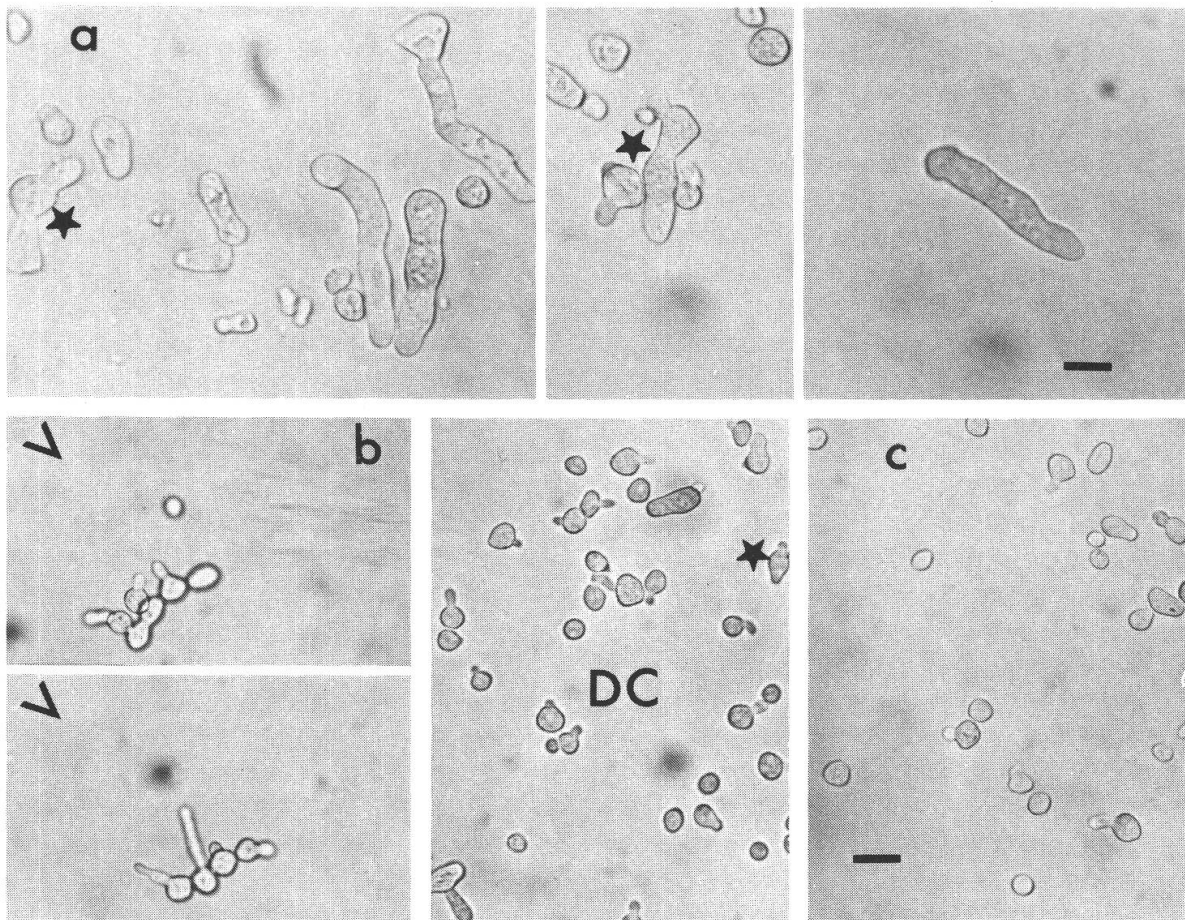


Fig. 1. a) Conidia of *Neurospora crassa* germinated in Vogel's liquid medium containing  $5 \cdot 10^{-4}$  M 2,4-DNP showing widened germ tubes and bipolar (\*) outgrowths. Bar = 10  $\mu$ m. – b) Germ tubes of *N. crassa* conidia chemotropically-oriented toward an increasing gradient (<) produced from crystals of 2,4-DNP asymmetrically seeded in a drop of liquid Vogel's medium flattened between microscope slide and cover slip. Bar = 10  $\mu$ m. – c) Stimulated rate of germ tube outgrowth (bipolar \*) in a suspension of conidia incubated at 20 °C in Vogel's liquid medium containing  $10^{-6}$  M dicoumarol (DC) compared to a control suspension (insert). Bar = 10  $\mu$ m.

nate by the more moderate concentration of  $10^{-5}$  M 2,4-DNP, while baso-lateral mitochondria remain well-developed (Fig. 2 b). At an intermediate concentration of the uncoupler ( $5 \times 10^{-4}$  M), the basis of germ tubes is frequently widened (Fig. 2 c) and there is frequent bipolar outgrowth (Fig. 1 in Turian 1980).

Complementarily to its uncoupling action, a protonophore such as 2,4-DNP could act as an external signal facilitating the intrusion of the proton current which specifies the site of outgrowth as proposed for the oriented emergence of the rhizoid observed in *Blastocladiella emersonii* (Harold and Harold 1980). In *N. crassa* conidia, 2,4-DNP can also function as an external signal to initiate the process of polar outgrowth of the germ tube(s) from conidia of *N. crassa*. We have observed them to respond chemotropically to 2,4-DNP by the positive orientation of their germ tubes toward the 2,4-DNP gradient (Fig. 1 b).

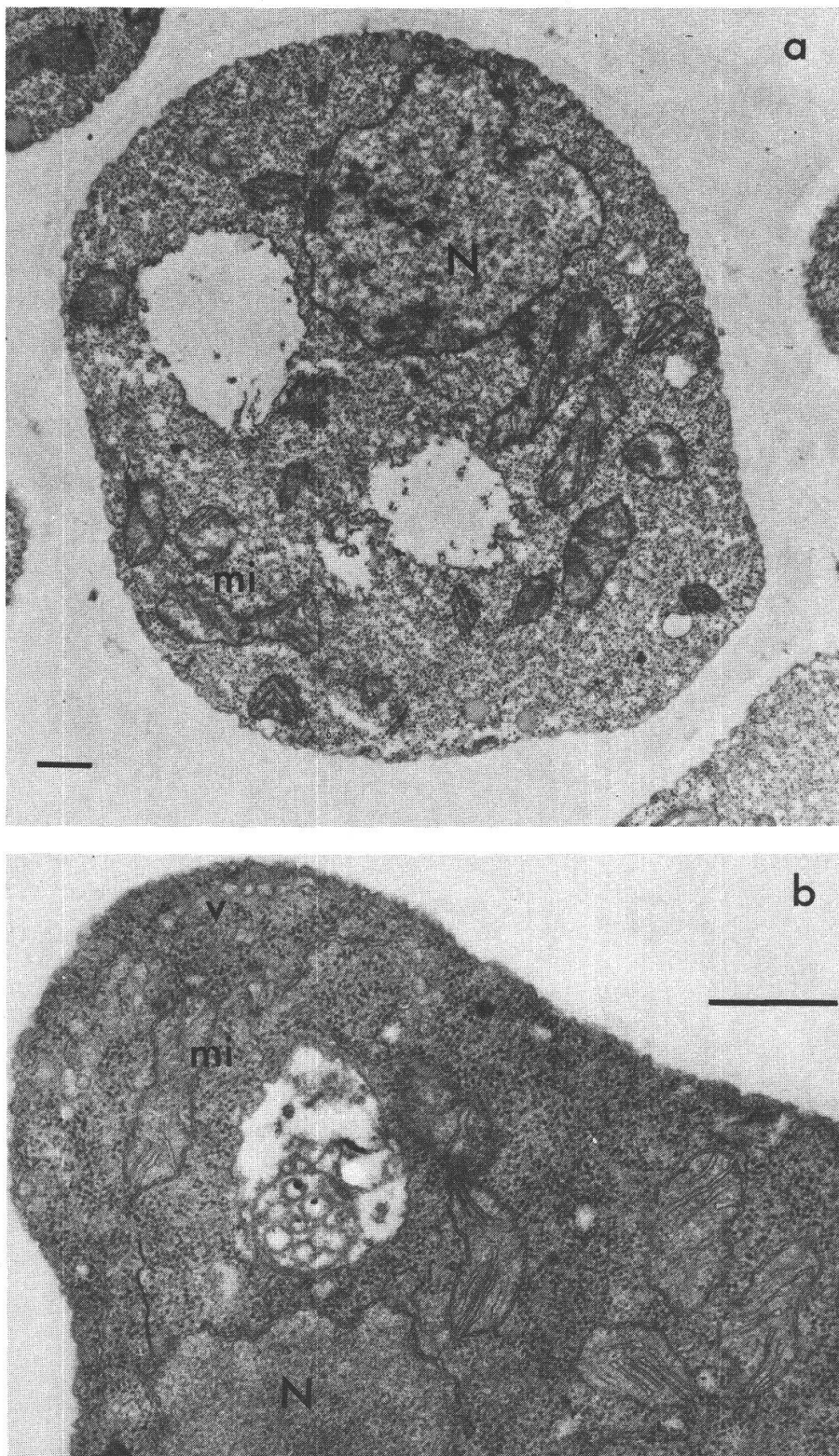


Fig. 2. Dinitrophenol (2,4-DNP)-treated conidia of *Neurospora crassa*. a)  $10^{-3}$  M in Vogel's liquid medium, 3 h at  $25^{\circ}\text{C}$ ; isometrically swollen conidium ( $14\ \mu\text{m}$  diameter instead of normal  $10\ \mu\text{m}$ ), mitochondria (mi) poorly differentiated and homogeneously dispersed in the cytoplasm. Nucleus (N). – b)  $10^{-5}$  M in Vogel's liquid medium, 2 h at  $25^{\circ}\text{C}$ ; poorly differentiated mitochondria (mi) lining the front of the outgrown germ tube. a=actin, ac=actomyosin, my=myosin, mt= microtubules, v= microvesicles, V= wall vesicles, Mi= mitochondrion. Bars =  $1\ \mu\text{m}$ .

### b) Cytochemical-functional features

To complement our ultrastructural study of mitochondrial defects in germinating conidia we have used cytochemical indicators. Cytochrome oxidase was first chosen because its complex structure can work as a redox-driven proton pump spanning the internal mitochondrial membrane (Wikström 1984). Practically, it can be detected with the 3,3'-diaminobenzidine (DAB) reaction that we have applied sequentially to the germination stages of *N. crassa* according to the method previously used (Ton-That et al. 1983). In short, outgrown tubes, the few mitochondria positioned frontally to the tip were found to be poorer in cytochrome oxidase than the subapical organelles (Fig. 3).

The cationic permeant probe Rhodamine 123, known to accumulate into mitochondria as a function of membrane potential (Johnson et al. 1981) fluoresced vividly in the group of subfrontal mitochondria of the fully quenched apex of the germ tube (Fig. 3 insert). Their apparently high membrane potential could result from their close contact with the highly protonized tip cytoplasm. As a consequence of their respiratory and coupling deficiencies, as well as their presumed permeation by protons, most frontal mitochondria are therefore expected to be less efficient in producing the ATP necessary for the functioning proton ATPase pumps located in the apical domes of elongating hyphae. It could be deduced from this that in the tips the inward leak of external protons would be dominant over their outward pumping. This proposal would be in agreement with the decreasing H<sup>+</sup> ATPase pumping activity measured toward the hyphal tips (Jennings 1979).

The electrogenic K<sup>+</sup> ionophore antibiotic valinomycin is a selective inhibitor (1 μM in Vogel's medium) of germ tube outgrowth (Turian et al. 1984). As K<sup>+</sup> ionophore it would intrude K<sup>+</sup> around the entire conidial plasmalemma, therefore antagonizing the leaked entry of H<sup>+</sup> through the selected site of germ tube outgrowth. As the protonophore 2,4-DNP, it dissipates the mitochondrial transmembrane potential (Johnson et al. 1981) which is then insufficient for ATP production. With both these ionophores, only a faint Rhodamine fluorescence was observed in mitochondrial granules of the swollen conidia.

The same inhibition of internal organization preparatory to outgrowth could be achieved with nigericin. This ionophore acts inversely than valinomycin by increasing the membrane potential of mitochondria (Johnson et al. 1981) by the promotion of electroneutral K<sup>+</sup>/H<sup>+</sup> exchanges (Harold 1986). In the presence of nigericin (5 μM), Rhodamine was brightly fluorescing in *all* mitochondrial granules of swollen conidia deprived of germ tubes.

### c) Preliminary biochemical criteria

The maintenance of hyphal growth in relatively high concentrations of 2,4-DNP (10<sup>-3</sup> M, in nitrate or 5.10<sup>-4</sup> M, in ammonium Vogel's medium; Vigne-Dvorniková and Turian, unpublished results 1980), which were even reduced in the length of their compartments by dense branching (colonial type of growth), confirm the statement of Clark-Walker (1973) that "oxidative phosphorylation is not required for apical hyphal growth". It should even be considered as "detrimental" to take in account the necessary segregation in the apical plasmamembrane of active proton leaks from ATP-requiring H<sup>+</sup> outpumping.

Nevertheless, the P/O ratio increases from its low value in dormant conidia (average 0.5, Ortega Perez et al. 1981) to higher values (1.0–1.7, Hall and Greenawalt 1964)

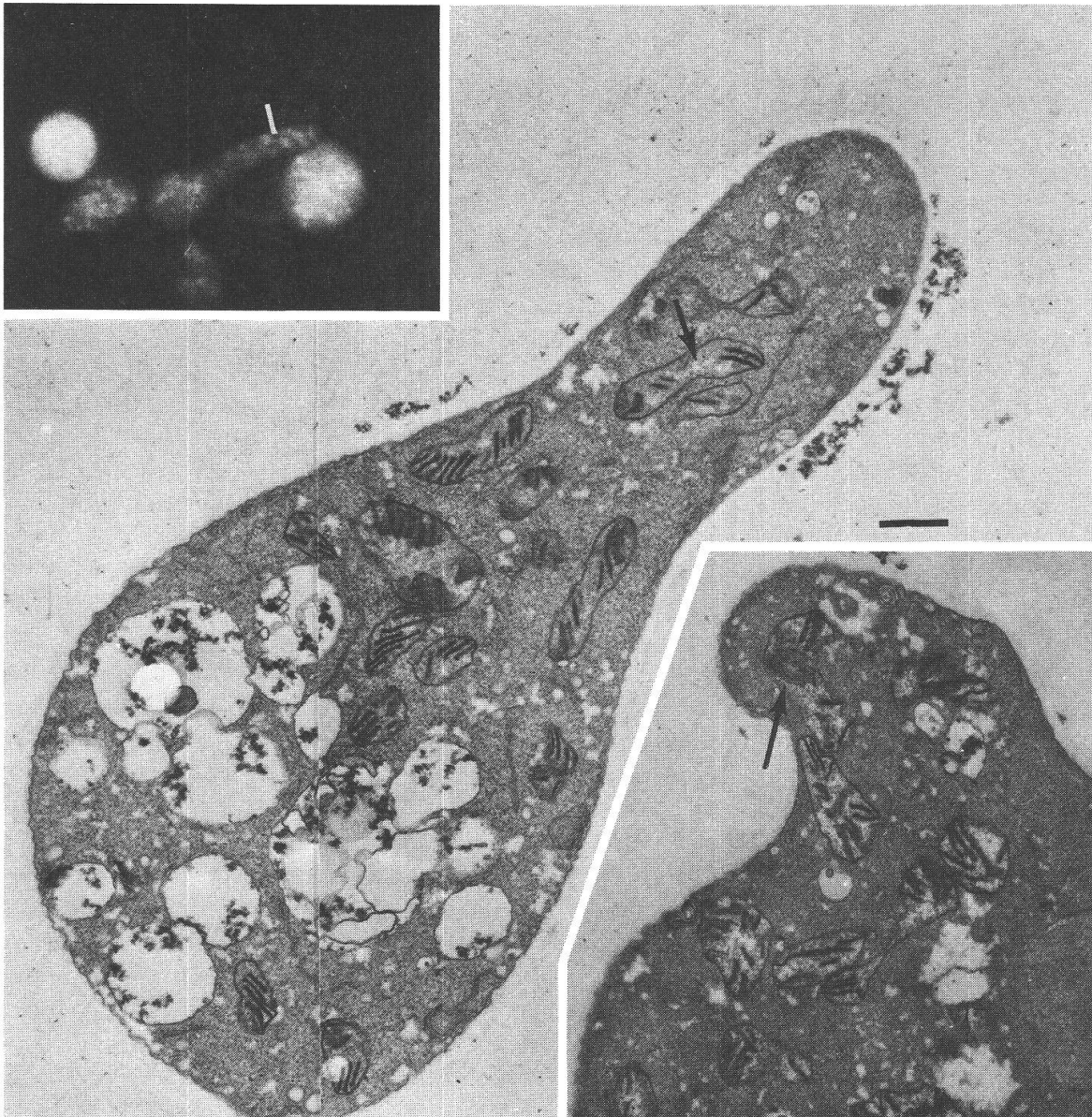


Fig. 3. Germinated conidia of *Neurospora crassa* after 2 h 30 at 25 °C in Vogel's liquid medium; DAB reaction for cytochrome oxidase in the mitochondria of germlings of *N. crassa*; restricted reaction in the underdeveloped frontal mitochondria (arrows) with only a few DAB positive cristae. Bar = 1  $\mu$ m. Insert: conidium in Vogel's liquid medium germinated for 3 h at 37 °C but finally incubated for 2 min in Rhodamine 123. Maximal greenish fluorescence = maximum membrane potential in the subapical mitochondria (dash). Bar = 5  $\mu$ m.

which are average values of *all* mitochondria isolated from hyphal apices and are therefore far from being maximal (3.0) in germinating conidia. Such energy evolution appears to be reflected in the ATP content of germinating conidia in which a slight drop was at first measured at the outgrowth stage (Ojha and Turian, unpublished results) when uncoupled frontal mitochondria are at their highest proportion compared to the coupled baso-lateral ones.



## 2. Genotypical effects

Primary signals from the genome impinge on the interwoven pattern of apical current and flow through membranar and cytoskeletal elements. This means primary genetical control over site selection of the outgrowing germ tube(s) (deficient mutants are known at this outgrowth stage, see Schmit and Brody 1976) as well over the number of germ tubes. As for this last criterium, germ tube outgrowth from conidia of *N. (Monilia) crassa* is essentially *monopolar* while it is mainly *bipolar* from those of *Monilia fructigena*. Consequently, even though only *one* outgrowth site per conidium is the standard pattern of germination in *N. crassa*, mutations may occur which depress such *monopolar* dominance.

We have now found such a multipolar germinating feature within the range of pleiotropic expression of the morphological, conditional conidial mutant "amycelial". Its conidia showed uncontrolled emergence of 2–6 (average 4) germ tubes (Fig. 4b), when they were incubated in liquid Vogel's medium modified with acetate-succinate as a single C source to be conidiation-permissive (Turian et al. 1971). On such a medium, germinated conidia of the wild type remained monopolar.

"Amycelial" is known to be respiratory-defective and poor in mitochondria (Oulevey et al. 1978). Its outgrown germ tubes are slow to elongate into vesicular hyphae. Such slow elongation growth could be related to the energetic deficiency of the mutant's mitochondria as expressed by their weak fluorescence in Rhodamine (Turian and Caesar 1987); their low membrane potential would reflect low ATP production and, consequently, low sustainment of the ATPase  $H^+$  outpumping with a low rate of  $H^+$  intrusion uniformly distributed among many germ tubes. The deficient mitochondria of "amyc" should therefore produce less of the ATP required to sustain plasmalemmal  $H^+$ -pumping ATPases while allowing  $H^+$  leaks leading to the multiple but moderately acidified sites of outgrowth. This multipolar effect could however additionally result from some genetical defect in the polarized assembly of the cytoskeleton, as recently found in the "multitube effect" of the antitubular agent benomyl on wild type conidia and their alleviation (recovery of monopolarity) in a benomyl-resistant mutant (Rossier et al. 1987; Caesar et al. 1988).

The genetically controlled multipolar pattern of germination could be phenocopied in wild type conidia germinated in the presence of ethylene glycol (Fig. 4b) in confirmation of Bates and Wilson's finding (1973). This effect of ethylene glycol might be ascribed to a higher mobility of the proton leaks, possibly due to an increased fluidity of the plasmalemmal lipoproteins, thereby facilitating lateral mobility of their proton channels and, consequently, allowing random positioning of multitubes.

## 3. Conclusions

Growing hyphae drive longitudinal proton currents through themselves (Harold et al. 1985, Harold 1986). These can be monitored either electrically (Kropf et al. 1983, 1984, Gow et al. 1984) or by evaluation of the pH gradient by staining indicators (Turian 1979, 1983) or by fluorescent probes (Turian 1981, 1983, Turian et al. 1985) along the apices of hyphae.

The pattern of electric current could be considered as a spatially extended chemi-osmotic system (Mitchell 1981) with proton pumps and proton leaks separated by as much as 300  $\mu m$  (Harold 1986). We also described (Turian 1980, Turian et al. 1985) polarized tip growth as implicating proton sources (frontal mitochondria) separated

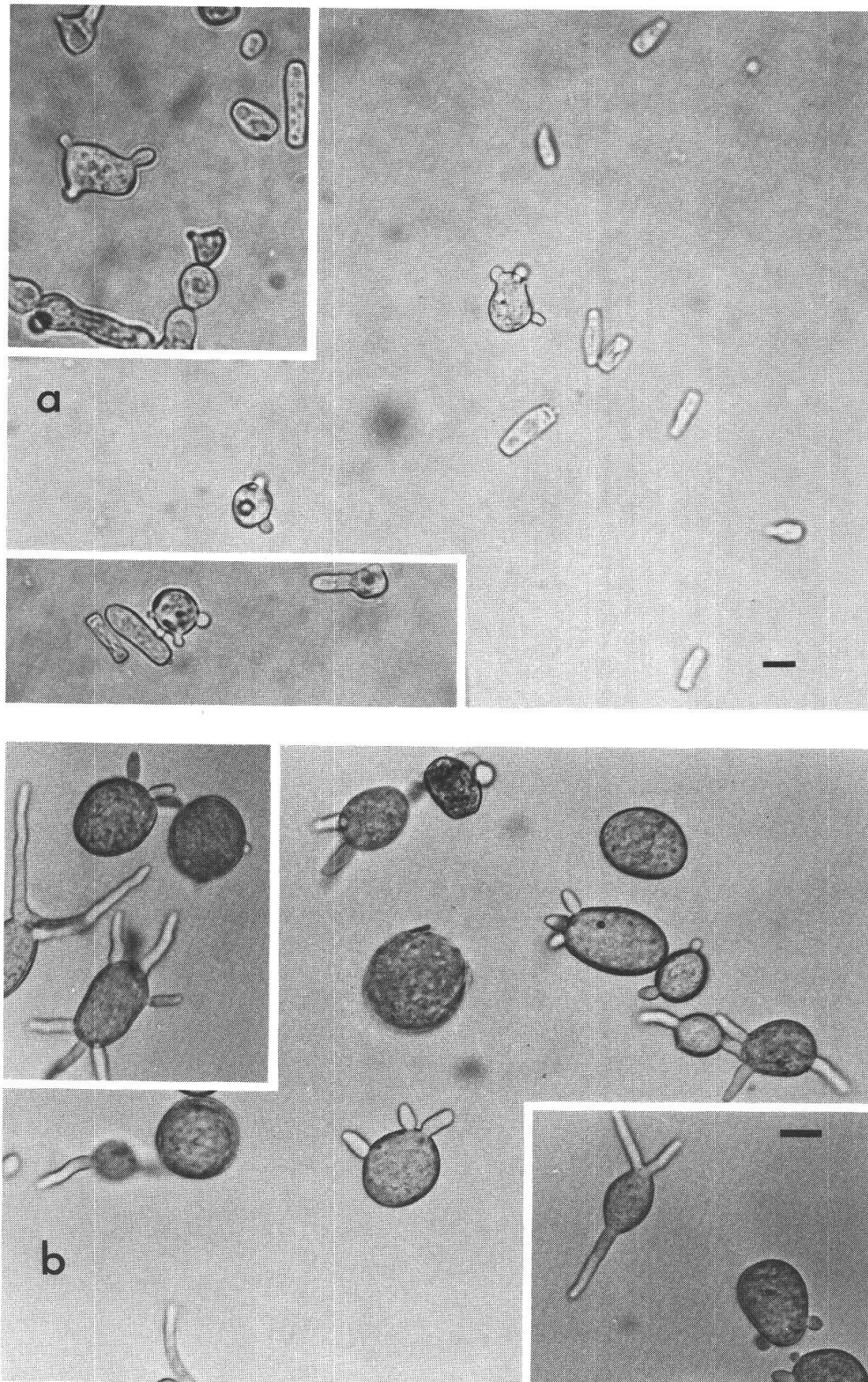


Fig. 4. a) Multipolar outgrowth of germ tubes from conidia of the morphological "amyc" mutant of *Neurospora crassa*. Bar = 5 µm. – b) Multipolar phenocopy of "amyc" by conidia of wild type *N. crassa* first germinated (1 h) in the presence of 3.22 M ethylene glycol and then stepped down to 0.322 M of the same compound for 2 h at 25 °C. Bar = 5 µm.

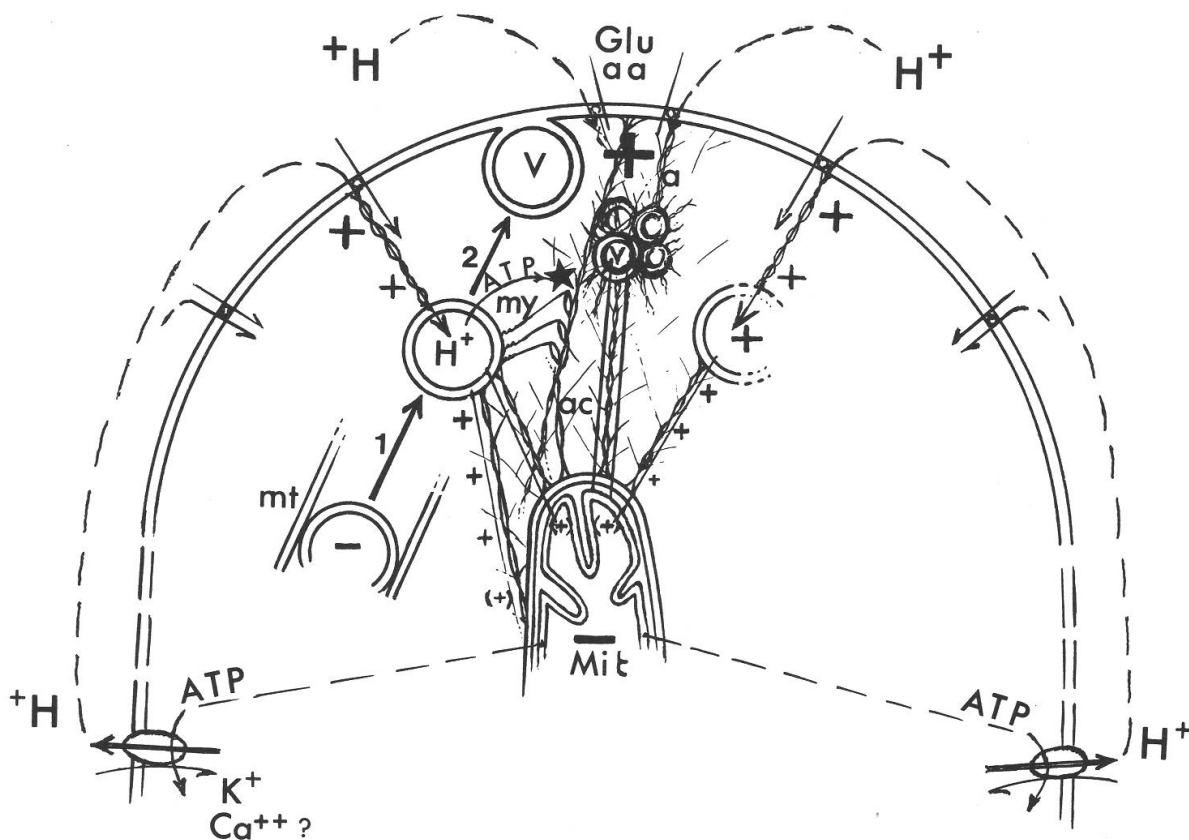


Fig. 5. Model (Turian et al. 1985) of proton circuitry in an hyphal tip updated to take in account a possible, natural or induced (2,4-DNP, DC, FCCP) uncoupled state of the frontal mitochondria (mit). Such mitochondria (minimally their frontal pole (+) as figured) would therefore be unable to fuel in ATP the H<sup>+</sup>-effluxing pumps (⊖) of their nearby tip plasmalemma; leak-channels (∩) at this level could therefore freely reintrude protons (acid tips; Turian 1981) in symport with glucose (glu) and/or amino acids (aa) (Harold 1986). – As previously proposed, the presumably negatively-charged wall vesicles could act as proton sinks and the energy of their electrochemical gradient has been hypothesized to be directly transduced into local synthesis of ATP to fuel the actin-activated myosin ATPase system (\*) which could be the driving force of acropetally moving vesicles. Continuous provision of ATP from subapical mitochondria and thereby continuous expelling and prospective reintrusion of protons would insure self-entrainment of this “proton motor” of tip growth.

from proton sinks (apical cytosol plus vesicles). Protons expelled from the hyphal trunk through H<sup>+</sup>-translocating ATPase (Slayman 1977) reach the growing tip by symport with amino acids or glucose (Harold 1982, Harold 1986). The distribution of the ATPases around the hyphal periphery is *not* yet specified but it may be expected that they are inefficient along the tip plasmalemma because of ATP deficiency due to possible P/O uncoupling in the frontal mitochondria (Turian and Geissler 1984). The depolarization of the outgrowth site could additionally help direct the apical clustering of H<sup>+</sup> channel-leaks and thus allow the entrance of the positive electric current loop. Thermodynamically, however, for net H<sup>+</sup> back pumping to occur through the leaks, counterion movement (anion influx or cation efflux) must dissipate the transmembrane potential. Consequently, after the initial, asymmetrical depolarizing burst of protons from the frontal mitochondria (see p. 135), vectorial acidification of the hyphal tips would continue to be insured, but indirectly, by the back-positioned, respiratory competent

mitochondria delivering their synthesized ATP to H<sup>+</sup>-effluxing ATPase of the proximal, subapical plasmalemma, thereby replenishing the external loops of positive current.

There is yet no experimental evidence as to whether individual mitochondria may have an overall electric polarity. Primary or secondary ion transport systems might be preferentially localized at one rather than both of their extremities. However, the suggestion has been made that the membrane of mitochondria could consist of a mosaic of domains of differential (high or low) conductance (Azzone et al. 1978). This therefore leaves open the possibility of polarity *initiated from inside* through local depolarization of the plasmalemma by protons asymmetrically effluxed from frontal, uncoupled mitochondria axiated along the genetically-controlled cytoskeleton toward the elected site(s) of germ tube(s) outgrowth.

Our new observations about the doubly defective ultrastructural and functional status of frontal mitochondria in outgrowing germ tubes and elongating hyphae, compared with the results of fungal electrophysiology (Harold 1986), lead us to reinterpret our model (Turian et al. 1985) of apical proton circuitry. The new scheme (Fig. 5) meets the requirements for spatial separation of H<sup>+</sup> pumps and channels (leaks) while maintaining mitochondria in their principal, although indirect, role of proton sources through ATP fueling of the plasmalemmal H<sup>+</sup> ATPase pumps.

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## Résumé

Les mitochondries de la pointe de l'unique tube germinatif émergeant des conidies de *Neurospora crassa* présentent une organisation ultrastructurale déficiente parallèle à leur potential de membrane réduit (testé par fluorescence de la Rhodamine 123) et une faible réaction DAB pour la cytochrome oxydase. Ces déficiences ultrastructurales sont généralisées à toutes les mitochondries de conidies ne pouvant que germer isométriquement sur des concentrations subléthales du découplant (protonophore) 2,4-dinitrophénol ( $10^{-3}$  M de 2,4-DNP) alors que les mêmes déficiences sont localisées dans les mitochondries frontales des tubes germinatifs élargis, parfois doubles, émergeant de conidies dans  $10^{-4}$  M de 2,4-DNP ou stimulées à germer normalement dans  $10^{-5}$  M de 2,4-DNP ou  $10^{-6}$  M d'un autre agent découplant, le dicoumarol. Le site d'émergence des tubes a pu être orienté dans un gradient de 2,4-DNP agissant probablement par dépolarisation membranaire locale et découplage. La valinomycine et la nigéricine ont été préliminairement testées pour leurs effets sur les potentiels de membrane des mitochondries de conidies inhibées dans leur germination. L'induction de germination multipolaire par l'éthylène glycol a pu être imitée génétiquement par les conidies produites conditionnellement par le mutant morphologique «amycelial». Les quelques mitochondries sur le front de leurs 4-6 tubes germinatifs n'ont que faiblement fluorescé dans la Rhodamine 123.

Notre précédent modèle de «moteur protonique» auto-entraînant les vésicules vers l'apex a été mis à jour pour tenir compte des conséquences des déficiences ultrastructurales et des déficiences respiratoires et de couplage des mitochondries frontales pour le circuit protonique régulé par l'ATP (pompes apicales «en panne», canaux ouverts) conduisant la croissance apicale.

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