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Short Communication

Antimycin A-resistant Respiratory Pathway in *Ustilago maydis* and *Neurospora sitophila*[1]

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Antimycin A inhibits electron transport at a site generally believed to be located between cytochromes *b* and *c* (10). However, certain respiratory systems such as those of *Rhodotorula glutinis* (8, 9) and *Symplocarpus foetidus* (skunk cabbage) (12) are insensitive to the antibiotic. Unestam and Gleason (13) showed that following an initial inhibition, antimycin A actually stimulates respiration of mycelia of a *Saprolegnia* sp. We have observed a similar phenomenon in conidia of *Neurospora sitophila* Shear and Dodge, treated with the antibiotic. Respiration of sporidia of *Ustilago maydis* (DeCandolle) Corda, on the other hand, is not inhibited initially by the antibiotic, but rather is immediately stimulated. A feature which distinguishes the respiration of antimycin A-treated cells of *N. sitophila* or *U. maydis* from that of untreated cells is the difference in sensitivity to oxygen tension. This report characterizes the antimycin A effects on respiration of these two fungi and also presents evidence that an alternate terminal oxidase operates in the cells after treatment with the antibiotic.

N. sitophila was grown at room temperature on agar medium (2) supplemented with 2 g/liter of yeast extract. Conidia from 4- to 7-day-old cultures were suspended in water or buffer solution (0.02 M phosphate containing 0.5 g/liter of $MgSO_4 \cdot 7H_2O$, pH 6.4), filtered through cheesecloth, and washed twice with water or buffer. Sporidia of *U. maydis* (ATCC 14826) were grown in shake culture at 30 C in a liquid medium of the same nutrient composition used to grow *N. sitophila*. After 18 to 24 hr, sporidia were centrifuged from the medium and washed twice with buffer solution. Unless otherwise specified, respiratory measurements were made with cells suspended in a glucose-mineral salts solution (pH6.4) supplemented with vitamins (2) but lacking a nitrogen source. In some experiments 0.25% (w/v) sodium acetate was substituted for glucose. Antimycin A was added in methanol. Final concentration of methanol in control and treated cultures did not exceed 1% (v/v). Cell suspensions were shaken at 25 or 30 C, and samples were withdrawn at appropriate intervals for measurement of oxygen consumption with a Gilson recording oxygen cathode (Oxygraph). Oxygen consumption under similar conditions was also measured manometrically. Levels of O_2 in the atmosphere differing from that in air were obtained by flushing the vessels for 15 min with O_2/N_2 mixtures containing 1, 2, or 5% O_2. Glucose and antimycin A were added from the sidearms after the vessels were closed and equilibrated. The KCN-KOH procedure of Robbie (11) was used for cyanide treatments in manometric experiments.

Oxygen uptake by conidia of *N. sitophila* utilizing glucose is inhibited about 75% after exposure for 30 min to 10 μg/ml of antimycin A. However, this inhibition declines, and by 3 hr the effect is one of respiratory stimulation. Respiration of sporidia of *U. maydis*, on the other hand, is immediately stimulated by the antibiotic (2–10 μg/ml). The stimulation persists for 5 hr or longer. A feature which distinguishes respiration of treated cells of either *U. maydis* or *N. sitophila* from that of untreated cells is a marked difference in sensitivity to low levels of oxygen. The rate of O_2 uptake by untreated sporidia of *U. maydis* is linear from solution saturation levels of O_2 (234 μM) to about 2% saturation. The rate of O_2 uptake by treated sporidia (utilizing glucose or acetate) begins to decline at about 20% saturation and declines quite rapidly at O_2 levels below 10% saturation (Figs. 1 and 2). Conidia of *N. sitophila* behave similarly, although a small decline in rate of O_2 uptake occurs in untreated conidia at O_2 levels above 2% saturation (Fig. 3).

The terminal oxidase of the rapid, cyanide-resistant respiration of *Arum spadix* tissue was reported to require a much higher O_2 concentration for saturation than the terminal oxidase of the slower, cyanide-sensitive respiration in other tissues of the same plant (5). However, Yocum and Hackett (14) concluded from their studies that at reduced O_2 tensions the diffusion rate of O_2 through the liquid-suspending medium, rather than a low affinity of the terminal oxidase for O_2, limits the respiration rate of aroid spadix tissue.

The following evidence indicates that increased sensitivity of respiration of antimycin A-treated cells of *N. sitophila* and *U. maydis* to O_2 tension is not due to O_2 diffusion limitations. The rate of O_2 uptake of treated cells falls below that of untreated cells at O_2 levels around 5% saturation for *U. maydis* and at about 4% for *N. sitophila* and then declines much more rapidly in treated than in the untreated cells. The rate of consumption by untreated sporidia of *U. maydis* at 2.5% O_2 saturation is 3 or 4 times that of treated sporidia even though the rate of consumption by the latter sporidia at higher levels of O_2 is more than 1.5 times that of the former. When glucose was omitted from suspensions of *U. maydis*, a marked stimulation of endogenous respiration was produced by antimycin A; however, the maximal rate of O_2 uptake at high levels of O_2 in solution was only 65% of that of untreated sporidia oxidizing glucose. Nevertheless, sensitivity of the respiration to O_2 tension was comparable to that of antimycin A-treated cells oxidizing glucose.

Respiratory rates at various oxygen concentrations were determined from slopes of lines drawn tangent to points along curves made with the Oxygraph. Accuracy of the method was increased by expanding sensitivity of the instrument so that the normal chart span was used to record respiratory rates at oxygen levels between 25 and 0% saturation. The reciprocal of velocity

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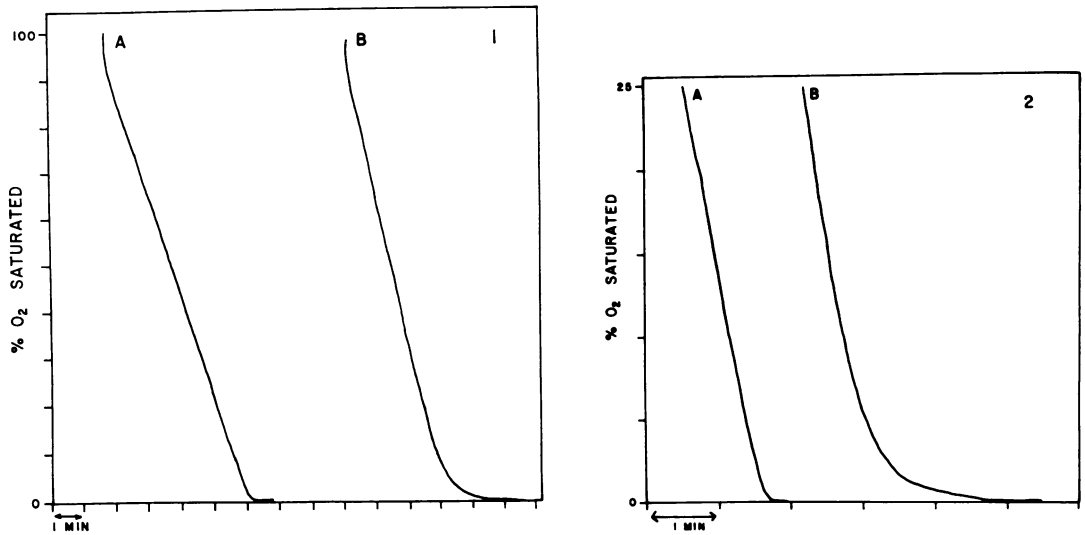

FIG. 1. Oxygen cathode recording showing rate of oxygen consumption (glucose substrate at 30 C) by sporidia of *Ustilago maydis* (0.7 mg dry weight per ml) at different levels of oxygen in solution. A: By untreated sporidia; B: By sporidia after incubation with 2 μg/ml of antimycin A for 45 min.

FIG. 2. Same as Figure 1, but with scales expanded. Sensitivity of instrument was adjusted so that recorder was off scale until sporidia reduced the oxygen level in solution to 25% of saturation.

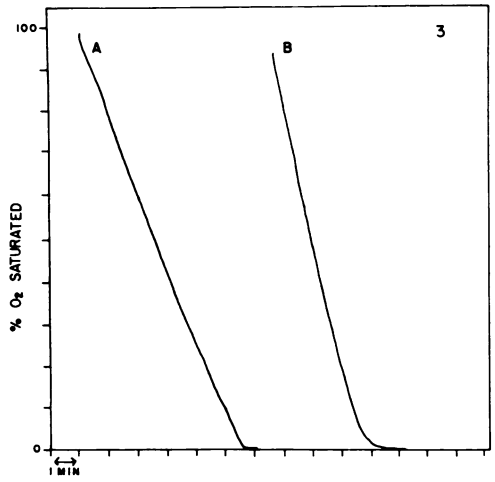

FIG. 3. Oxygen cathode recording showing rate of oxygen consumption (glucose substrate at 30 C) by conidia of *Neurospora sitophila* (6.8 mg dry weight per ml) at different levels of oxygen in solution. A: By untreated conidia 3 hr after addition of substrate; B: By treated conidia 3 hr after addition of substrate and 10 μg/ml of antimycin A.

was plotted against the reciprocal of O_2 concentration, and the O_2 concentration-supporting half-maximal respiratory rate (K*m*) was calculated. The K*m*-values for *U. maydis* sporidia treated with 2, 4, or 10 μg/ml of antimycin A ranged from 4.5 to 6% O_2 saturation, whereas those for untreated sporidia ranged from 0.5 to 0.6%. The values for treated and untreated conidia of *N. sitophila* were 5 and 0.6%, respectively. The O_2 molarity values at K*m*, which are presented in Table I, show that both *N. sitophila* and *U. maydis* are nearly 10 times as sensitive to O_2 tension in the presence of antimycin A as in its absence.

The effect of O_2 level on respiration of *N. sitophila* and *U. maydis* was also determined manometrically. Results were consistent with those obtained in polarographic measurements. In an atmosphere of air at 25 C the Q_{O_2} was 19 and 11, respectively, for treated (10 μg/ml) and untreated conidia of *N. crassa*. At an atmospheric level of 0.9% O_2, the Q_{O_2} for treated conidia was 7.6, whereas at a level of 0.75% O_2, the Q_{O_2} for untreated conidia was

Table I. *Oxygen Concentration-supporting Half-maximal Respiratory Rate (Km) in Antimycin A-treated and Untreated Cells of Ustilago maydis and Neurospora sitophila*

Organism	O_2 concn (μM)	
	Treated	Untreated
U. maydis	11–14	1.2–1.4
N. sitophila	12	1.4

10.5. The Q_{O_2} in an atmosphere of air at 30 C was 143 and 79, respectively, for treated (2 μg/ml) and untreated sporidia of *U. maydis*. The Q_{O_2} at approximately 1% O_2 in the atmosphere was 63 and 80, respectively, for treated and untreated sporidia.

Respiration of the two fungi was resistant to cyanide. The Q_{O_2} of *U. maydis* sporidia was increased from 82 to 143 by 4.6×10^{-5} M HCN. A concentration of 4.6×10^{-4} M HCN produced a similar stimulation of O_2 uptake for about 1.5 hr, but at the end of 3 hr the rate was about equal to that of the control. Respiration of *N. sitophila* was inhibited about 50% after 1 hr by 4.6×10^{-4} M cyanide, but after 3 hr there was little or no inhibition. No tests were made to determine whether the cyanide-resistant respiration was similar to antimycin A-resistant respiration in sensitivity to O_2 tension.

The respiratory stimulation and increased sensitivity to O_2 tension which follows antimycin A treatment is apparently the consequence of a shift in the pathway of electron transport to an alternate terminal oxidase. Appreciable evidence indicates that antimycin A specifically blocks electron transport between cytochromes *b* and *c* (1, 3, 6). Therefore, electrons are probably diverted to an alternate oxidase at a site preceding cytochrome c. Spectroscopic examination of *U. maydis* sporidia showed that cytochrome b remains reduced in the presence of antimycin A, whereas cytochromes *c* and a_3 are oxidized (4). The alternate pathway is apparently of considerable value to *U. maydis* for growth in the presence of the antibiotic because growth of a mutant lacking the system is much more sensitive to antimycin A than that of the wild type (4). The antimycin A-resistant respira-

tion of *U. maydis* and *N. sitophila* resembles that described for *Rhodotorula glutinis* (8, 9), skunk cabbage mitochondria (12), and a *Saprolegnia* sp. (13). The systems appear to differ somewhat from the cyanide-resistant respiratory system of *Myrothecium verrucaria* (7).

Stimulation of respiration in *U. maydis* by cyanide probably results from a shift in pathway of electron transport which by-passes phosphorylation sites rather than from a typical uncoupling such as that produced by 2,4-dinitrophenol. It seems likely, at least in *U. maydis*, that the same alternate electron transport pathway is utilized when either cyanide or antimycin A is present. Such is reported to be the case in skunk cabbage mitochondria (12).

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