Alternative Cyanide- and Antimycin A-insensitive Respiratory System in *Sporobolomyces* Red Yeasts

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Sporobolomyces ruberrimus is insensitive to antimycin A which is a respiratory inhibitor of the cytochrome system, as cyanide is. When this red yeast was cultured in the presence of antimycin A, the growth curve showed the same pattern as that of the normal culture in the absence of it, but the growth mass was only about 70% of that of the normal culture. The antimycin A-insensitive and cyanide-insensitive respiration of *Sp. ruberrimus* was inhibited by pyrocatechol and salicylhydroxamic acid. *Sporobolomyces* red yeasts have two characteristic terminal oxidase systems; one is a cytochrome oxidase system and the other is a cyanide- and antimycin A-insensitive oxidase system. The proportions of the two respiratory systems differed among the species and strains of *Sporobolomyces* red yeasts examined.

The functions of alternative cyanide-insensitive respiratory systems have already been discussed, and a scheme was proposed for the fungus, *Neurospora crassa*.¹⁾ As previously reported,²⁾ a similar respiratory system is present in *Sp. ruberrimus*, that is not inhibited by cyanide or carbon monoxide. This yeast cyanide-insensitive respiratory pathway was inhibited by *o*-diphenol derivatives and salicylhydroxamic acid (SHAM).

In this paper, we report that cyanide-insensitive respiration is widespread among *Sporobolomyces* red yeasts. Furthermore, we describe the effect of antimycin A on the cyanide (CN)-sensitive and -insensitive respirations.

MATERIALS AND METHODS

Microorganisms. *Sporobolomyces ruberrimus*³⁾ and other red yeast strains stocked in our laboratory were used in this experiment.

Oxygen uptake. Oxygen uptake was measured by the Warburg manometric technique at 25°C. The results obtained were expressed as Q_{O_2} values. The values given in this paper are the ratios to control Q_{O_2} values for autorespiration and glucose oxidation.

Medium. The strains used were cultured in the medium at 25°C for 2 days and rested as described previously.⁴⁾

Reagents. Antimycin A (Sigma Chemical Co.) was dissolved in absolute ethanol (mg/ml) and diluted with distilled water when used.

Cultures in the presence of antimycin A. 1 μ g or 10 μ g of antimycin A per ml (final concentration) was added to the medium as described previously.⁴⁾ *Sp. ruberrimus* was inoculated into 50 ml of the medium with antimycin A in a 500 ml shaking flask and incubated at 25°C with reciprocal shaking. The weight of the growing cells of *Sp. ruberrimus* was determined at 12 hr intervals.

AM-cells. *Sp. ruberrimus* cells were incubated in the above medium containing 1 μ g of antimycin A per ml (final concentration) at 25°C for 48 hr with shaking and then rested in 0.2 M phosphate buffer (pH 5.8) containing 1 μ g of antimycin A per ml for another 24-hr at 25°C with shaking. The cells thus prepared were called AM-cells.

Other experimental methods were the same as described previously.²⁾

RESULTS

Antimycin A is known as a respiratory inhibitor of cytochrome systems, as cyanide is. *Sp. ruberrimus* was investigated as to whether it exhibited insensitive respiration in the presence of antimycin A, as in the presence of

FIG. 1. Effect of Antimycin A on Respiration of *Sporobolomyces ruberrimus*.

When antimycin A was used, it was dissolved in a very small volume of ethanol (1 mg/ml) and diluted with distilled water ($\mu\text{g/ml}$). Each manometer vessel contained 1 ml of a cell suspension in 0.2M phosphate buffer (pH 5.8), reagent(s) and distilled water to bring the volume to 2.0 ml, and 0.3 ml of 10% of KOH was placed in the center well. The total volume was 2.3 ml. ○—○, no addition; △—△, 0.2 ml of 1% ethanol; ●—●, 0.5 ml of 4% glucose; □—□, 0.2 ml of 10 $\mu\text{g/ml}$ antimycin A; ▲—▲, 0.2 ml of 10 $\mu\text{g/ml}$ antimycin A and 0.5 ml of 4% glucose.

cyanide, or not. As shown in Fig. 1, the respiration of *Sp. ruberrimus* was not inhibited at all, but, in fact, it was moderately accelerated by antimycin A, exactly as in the case of cyanide. But this antimycin A (AM)-insensitive respiration was almost completely inhibited by the addition of pyrocatechol, as in the case of CN-insensitive respiration (Fig. 2). Therefore, the alternative respiratory system of *Sp. ruberrimus* was suggested to be as shown in Fig. 3, which Lambowitz *et al.*⁵⁾ proposed for the *poky* mutant mitochondria of the *N. crassa* respiratory system.

Whether or not this AM-insensitive respiratory system is physiologically active in *Sp. ruberrimus* was examined. *Sp. ruberrimus* was

FIG. 2. Effects of Antimycin A and/or Pyrocatechol on Autorespiration of *Sporobolomyces ruberrimus*.

The conditions were the same as in Fig. 1. △—△, no addition; ●—●, 1 $\mu\text{g/ml}$ antimycin A; ○—○, 1 $\mu\text{g/ml}$ antimycin A and, after 30 min, 10 mM pyrocatechol was added; □—□, 1 $\mu\text{g/ml}$ antimycin A and 10 mM pyrocatechol.

The arrow indicates the time of addition of pyrocatechol.

FIG. 3. Branched Electron Transport System.

Y denotes the branch point and X denotes the cyanide- and antimycin A-insensitive oxidase system, according to Lambowitz *et al.*⁵⁾ The sites of inhibition by inhibitors are indicated by dashed lines.

SHAM, salicylhydroxamic acid; CAT, pyrocatechol.

inoculated into a liquid medium containing 5% sucrose, 0.5% peptone, 0.1% KH_2PO_4 , 0.1% NaNO_3 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 μg or 10 μg of antimycin A per ml (pH 5.8), cultivated at 25°C with shaking and then sampled at 12 hr intervals to determine the cell

FIG. 4. Effect of Antimycin A on the Growth of *Sporobolomyces ruberrimus*.

Sporobolomyces ruberrimus was grown in a liquid medium containing 5.0% sucrose, 0.5% peptone, 0.1% KH_2PO_4 , 0.1% NaNO_3 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and antimycin A at 25°C with shaking.

○—○, control; △—△, 1 µg of antimycin A per ml; ●—●, 10 µg of antimycin A per ml; □—□, 1 µg of antimycin A and 0.2 mg of SHAM per ml.

Refer to Fig. 1 for other conditions.

weight (Fig. 4). *Sp. ruberrimus* grew in the presence of 1 µg or 10 µg antimycin A, although the cell weights were less than in the absence of antimycin A, and the growth curve with antimycin A showed a striking resemblance to the normal growth curve. The coexistence of antimycin A and SHAM, which did not effect the respiration of this yeast individually, strongly inhibited the growth of the yeast. Consequently, it is possible that this insensitive respiratory system is physiologically active in *Sp. ruberrimus* but with an inferior efficiency as to glucose oxidation, different from in the case of the normal respiratory system.

As previously reported,^{2,4)} on carbohydrate utilization this red yeast oxidizes 3 M of 6 M glucose and assimilates the other 3 M in the presence of cyanide. The glucose utilization of AM-cells, which were cultured in the presence of antimycin A, was compared with that of normal cells in the presence of cyanide. As shown in Fig. 5, the efficiency of glucose oxidation in the former cells is the same as that in the latter cells with cyanide but is somewhat inferior to that in normal cells.

Also, the AM-insensitive respiration in AM-cells was inhibited by SHAM, as well as pyrocatechol.

FIG. 5. Balance of Glucose Consumption and Oxygen Uptake in Normal Cells and AM-Cells.

Sporobolomyces ruberrimus was grown in 100 ml of the medium used in Fig. 4, containing 1 ml of 100 µg of antimycin A per ml in 10% ethanol, at 25°C for 2 days with shaking. The cells were harvested by centrifugation, and then washed three times with 0.9% saline. They were resuspended in 0.2 M phosphate buffer (pH 5.8) with 1 µg of antimycin A per ml in 0.1% ethanol and shaken for another 24-hr at 25°C. The cells thus obtained were called AM-cells. The other conditions were the same as in Fig. 1 except that 1 ml of the cell suspension was placed in a side arm. Normal cells: I, no addition; II, 5 µM glucose; III, 5 µM glucose and 2 mM NaCN, AM-cells: IV, no addition; V, 5 µM glucose.

TABLE I. EFFECT OF NaCN ON THE RESPIRATION OF VARIOUS *Sporobolomyces* SPECIES AND STRAINS

Strain	Autorespiration 2 mM NaCN		Glucose oxidation 2 mM NaCN	
	— Q _{O₂}	+ Ratio	— Q _{O₂}	+ Ratio
<i>Sporobolomyces alborubescens</i> IFO 1031	5.98	130	29.07	51
<i>Sp. odor</i> IFO 1035	2.71	163	26.66	25
<i>Sp. odor</i> IFO 1606	3.27	216	16.32	125
<i>Sp. roseus</i> *	4.35	213	33.65	82
<i>Sp. roseus</i> IFO 1105	6.01	167	31.29	44
<i>Sp. pollacii</i> IFO 1037	9.94	156	23.87	100
<i>Sp. tenuis</i> IFO 1040	6.39	219	18.46	107
<i>Sp. holsaticus</i> IFO 1034	3.27	171	9.52	116
<i>Sp. holsaticus</i> IFO 1032	5.71	233	31.92	72
<i>Sp. pararoseus</i> IFO 1103	8.94	89	24.24	33
<i>Sp. pararoseus</i> IFO 0376	9.33	209	39.96	52
<i>Sp. pararoseus</i> IFO 1104	8.54	209	16.08	104
<i>Sp. pararoseus</i> IFO 1107	8.17	170	44.56	106
<i>Sp. salmonicolor</i> *	6.67	20	22.21	8
<i>Sp. salmonicolor</i> IFO 0375	5.70	16	27.21	5
<i>Sp. ruberrimus</i> *	7.00	227	16.02	147
<i>Sp. ruberrimus</i> var. <i>albus</i> *	7.45	187	10.61	201

$$\text{Ratio} = \frac{Q_{O_2} \text{ with NaCN}}{Q_{O_2} \text{ without NaCN}} \times 100. \quad *, \text{ Refer to ref. 3.}$$

Other Sporobolomyces yeasts. Then, *Sporobolomyces* red yeasts other than *Sp. ruberrimus* were investigated as to the alternative respiratory system. As shown in Table I, the oxygen uptake of various red yeast strains with and without cyanide was expressed as Q_{O₂} values for autorespiration and glucose oxidation. As to autorespiration, the *Sporobolomyces* red yeasts tested showed increasing respiration as in the case of *Sp. ruberrimus*, except for *Sp. pararoseus* and *Sp. salmonicolor*. The rate of the accelerated oxygen uptake differed according to the species and strains tested. Consequently, it is possible that these *Sporobolomyces* red yeasts have two respiratory systems, that is, one is a normal cytochrome oxidase system and the other is an alternative respiratory system.

As to glucose oxidation, *Sp. holsaticus* IFO 1032, *Sp. alborubescens*, *Sp. pararoseus* IFO 0376, *Sp. roseus* and *Sp. roseus* IFO 1105 showed slightly inhibited respiration, different from in the case of the accelerated oxygen

uptake on autorespiration. *Sp. odor*, *Sp. pararoseus*, *Sp. salmonicolor* and *Sp. salmonicolor* IFO 0375 showed remarkable inhibition.

The effects of cyanide, antimycin A, pyrocatechol and SHAM on the respiration of *Sporobolomyces* red yeasts were investigated too. The results are presented in Table II. The values shown in the Table are the ratios to the Q_{O₂} values without reagent for autorespiration and glucose oxidation. The respiration of the *Sporobolomyces* red yeasts tested was accelerated by antimycin A, similar to in the case of cyanide, for autorespiration and glucose oxidation, but that of *Sp. salmonicolor* IFO 0375 was considerably inhibited in both cases. When the tests were performed in the presence of NaCN or antimycin A and pyrocatechol or SHAM, the results differed with the species or strains tested.

As to autorespiration, *Sp. holsaticus* IFO 1032 and *Sp. odor* IFO 1606 did not show complete inhibition on the addition of NaCN and SHAM or pyrocatechol. As to glucose

TABLE II(a). EFFECTS OF INHIBITORS ON AUTORESPIRATION OF *Sporobolomyces* SPECIES AND STRAINS

	Auto	CN	AM	SHAM	CAT	CN + SHAM	CN + CAT	AM + SHAM	AM + CAT	AM + CN	SHAM + CAT
<i>Sp. ruberrimus</i>	100 (5.96)	237	251	123	148	23	0	33	25	259	126
<i>Sp. pararoseus</i> IFO 0376	100 (9.33)	209	200	143	194	17	20	13	12	213	266
<i>Sp. pararoseus</i> IFO 1107	100 (8.17)	170	364	164	191	6	26	7	9	195	155
<i>Sp. pararoseus</i> IFO 1104	100 (8.54)	209	421	127	132	10	4	9	0	393	162
<i>Sp. holsaticus</i> IFO 1032	100 (5.71)	233	320	186	540	71	66	83	64	101	314
<i>Sp. salmonicolor</i> IFO 0375	100 (5.70)	16	24	126	573	20	28	48	57	13	211
<i>Sp. roseus</i> IFO 1105	100 (6.01)	167	271	114	309	11	8	26	18	211	188
<i>Sp. odorus</i> IFO 1606	100 (3.27)	216	910	246	469	92	82	196	202	831	702

All values are ratios to the values for autorepiration.

The values in parentheses are the Q_{O_2} values for autorepiration.

TABLE II(b). EFFECTS OF INHIBITORS ON GLUCOSE OXIDATION OF *Sporobolomyces* SPECIES AND STRAINS

	Glucose	CN	AM	SHAM	CAT	CN + SHAM	CN + CAT	AM + SHAM	AM + CAT	AM + CN	SHAM + CAT
<i>Sp. ruberrimus</i>	100 (20.25)	149	191	115	103	14	13	28	9	164	108
<i>Sp. pararoseus</i> IFO 0376	100 (39.96)	52	91	99	96	5	3	6	4	83	109
<i>Sp. pararoseus</i> IFO 1107	100 (44.56)	106	159	99	83	20	2	78	4	47	18
<i>Sp. pararoseus</i> IFO 1104	100 (48.08)	104	141	106	92	5	3	6	6	117	93
<i>Sp. holsaticus</i> IFO 1032	100 (31.92)	72	65	103	121	37	12	12	6	58	118
<i>Sp. salmonicolor</i> IFO 0375	100 (27.21)	5	18	111	140	5	8	18	24	4	123
<i>Sp. roseus</i> IFO 1105	100 (31.29)	44	41	99	99	11	11	9	5	20	92
<i>Sp. odorus</i> IFO 1606	100 (16.32)	125	141	172	202	15	9	39	40	90	207

All values are ratios to the values for glucose oxidation.

The values in parentheses are the Q_{O_2} values for glucose oxidation.

oxidation, all strains tested were not affected by pyrocatechol or SHAM but were inhibited when they coexisted with NaCN or antimycin A.

From the results described above, it can be

assumed that *Sporobolomyces* red yeasts have two respiratory systems, a cytochrome oxidase system and a CN- and AM-insensitive respiratory system, and the proportions of these two respiratory systems in *Sporobolomyces*

differ with the species or strains.

DISCUSSION

The respiration of *Sp. ruberrimus* was insensitive to antimycin A as well as cyanide. Also, the AM-insensitive respiration was inhibited by pyrocatechol and SHAM, as the CN-insensitive respiration was. That this AM-insensitive respiration physiologically occurs in *Sp. ruberrimus* was confirmed by the results of the growing culture test with antimycin A. Since the growth mass in the presence of antimycin A was less than that of normal cells, the efficiency of the alternative respiratory system as to glucose oxidation is very inferior compared to the cytochrome oxidase system (Fig. 4).

Also, as to the glucose that disappeared in AM-cells, 3 M of 6 M glucose was oxidized and the other 3 M was assimilated as in the case of CN-insensitive respiration. The utilization of glucose by AM-cells is very inferior compared to that of normal cells (Fig. 5).

All *Sporobolomyces* strains tested, except

Sp. salmonicolor, have both CN- and AM-insensitive respiration and a cytochrome oxidase system like *Sp. ruberrimus*. It may be assumed that the proportions of the two respiratory systems differ according to the species or strains, respectively.

The insensitive respiratory chains in cell free system will be reported elsewhere. This problem still requires further investigation.

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