[Chem. Pharm. Bull. 26(10)2971—2976(1978)]

UDC 576.852.1.097.098 : 615.277.4.015.4.076.7

Isolation of Antimycin-resistant Variants of *Candida utilis*

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(Received February 18, 1978)

In the process of isolating several antimycin-resistant mutants of *Candida utilis* by treatment with N-methyl-N′-nitro-N-nitrosoguanidine, we found a simple method of isolating many antimycin-resistant variants without treatment with mutagen. When cells were grown in glucose medium containing 10^{-6} M antimycin for more than three days at 30°, we observed the existence of some antimycin-resistant variants in the culture medium. These variants can grow in glycerol medium containing 10^{-6} M antimycin in which the parental strain can not grow.

The results of fluctuation test strongly suggested that antimycin-resistant variants arose unperiodically by spontaneous mutation.

The respiration of antimycin-resistant variants was not inhibited by 10^{-7} M antimycin which inhibited completely that of parental strain, but was inhibited by the higher concentrations of antimycin. Therefore, these variants do not seem to become antimycin resistant by the possession of any special antimycin-resistant respiratory chain as reported on several fungi. Some other possibilities were discussed.

Keywords——isolation; antimycin; drug resistance; *Candida utilis*; fluctuation test; growth inhibition; respiratory activity

Antimycin has been widely used as an inhibitor of the respiratory chain.[2)] We succeeded in isolating antimycin-resistant mutants of *Candida utilis* by treatment with N-methyl-N′-nitro-N-nitrosoguanidine in an attempt to elucidate the mechanism of inhibitory effects of antimycin. In this process, we found a simple method for isolating antimycin-resistant variants without treatment with mutagen.

We describe here the new method and some properties of these antimycin-resistant variants.

Experimental

Chemicals——Antimycin (Sankyo Co. Ltd.) was dissolved in absolute ethanol to make 10^{-3} M solution, and stored at 5°.

Microorganism, Media and Growth Conditions——*Candida utilis* 0619 was grown aerobically at 30° in Ogur[3)] medium. Glucose was used as a fermentable carbon source and glycerol as a non-fermentable one. Glucose medium is referred to a medium containing glucose and glycerol medium to a medium containing glycerol. The growth curve was obtained from the optical density of 5-fold dilution of the culture at 660 nm with a Hiranuma electric photometer (EPO-B).

Method for isolating Antimycin-resistant Variants——Cells were grown on nutrient agar (glucose medium) for 24 hr. One loopful of grown cells was inoculated into 10 ml of the glucose medium containing 10^{-6} M antimycin and cultured for more than 72 hr with reciprocal shaking. The culture medium was diluted adequately and plated on glycerol medium containing 10^{-6} M antimycin in which resistant cells could form colonies and sensitive cells could not.

Measurement of Respiratory Activities——Respiratory activities of cells were measured at 30° by the conventional manometric procedures of Warburg.

1) Location: *Oshika 2-2-1, Shizuoka-shi, 422, Japan.*
2) E.C. Slater, *Biochim. Biophys. Acta*, **301**, 129 (1973).
3) M. Ogur and R. St. John, *J. Bacteriol.* **72**, 500 (1956).

Results

Effects of antimycin on growth of *Candida utilis*

Cells were grown aerobically in glucose medium or glycerol medium containing various concentrations of antimycin. Growth curves shown in Fig. 1 were obtained from the measurement of turbidities of these culture media.

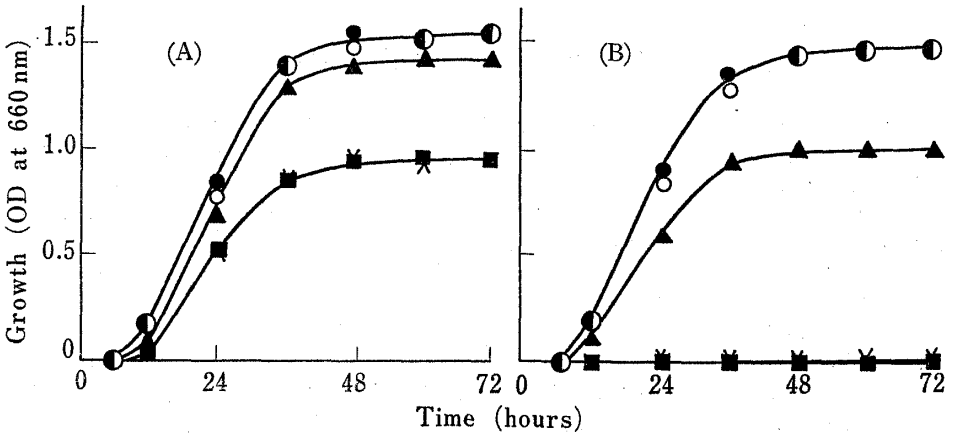

Fig. 1. Effects of Antimycin on Growth of *Candida utilis* 0619

Candida utilis 0619 was inoculated into glucose medium (A) or glycerol medium (B) containing various concentrations of antimycin and cultured at 30° with reciprocal shaking. Optical densities were measured in a Hiranuma electric photometer (EPO-B) fitted with a No. 66 filter. ●—●, control; ○—○, with 10^{-8} M; ▲—▲, with 10^{-7} M; ×—×, with 10^{-6} M; ■—■, with 10^{-5} M antimycin.

In glycerol medium, antimycin inhibited the growth of *C. utilis* at the concentrations higher than 10^{-6} M and partially inhibited the growth at 10^{-7} M antimycin. But in glucose medium, the growth was not completely inhibited at the concentrations higher than 10^{-6} M

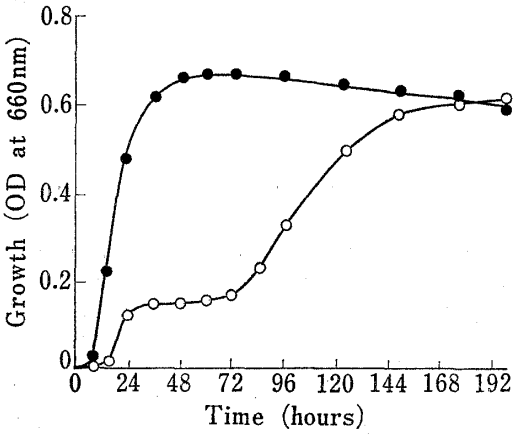

Fig. 2. Effects of Antimycin on Growth of *Candida utilis* 0619

Candida utilis 0619 was inoculated into glucose medium (●—●) or glucose medium containing 10^{-6} M antimycin (○—○) and cultured at 30° with reciprocal shaking. Culture medium was taken and diluted to 1/5. Optical density of the diluted culture medium was measured at 660 nm.

Table I. Emergence of Antimycin-resistant Variants

Culture time (hr)	Number of antimycin-resistant variants Cutlured in Glucose medium $+10^{-6}$ M antimycin	Glucose medium —
0	0	0
24	0	0
48	0	0
56	0	0
60	3	0
62	12	0
64	30	0
66	33	0
68	70	0
72	>100	0
96	>100	0

One loopful of cells was inoculated into L-shaped tubes containing 10 ml of culture medium with or without 10^{-6} M antimycin and grown at 30° with reciprocal shaking. One loopfuls of culture media were taken and streaked on glycerol medium containing 10^{-6} M antimycin. The number of colonies was counted after incubation for 72 hr at 30°.

antimycin. This result indicates that the inoculated cells were all sensitive to antimycin and could not utilize non-fermentable carbon source under the condition where the respiratory chain was blocked by antimycin.

Growth in Glucose Medium containing 10^{-6} M Antimycin and Emergence of Antimycin-resistant Variants

Cells were grown in glucose medium with or without 10^{-6} M antimycin.

As shown in Fig. 2, typical growth curve was obtained when *C. utilis* was grown in glucose medium without antimycin. But when *C. utilis* was grown in the presence of antimycin, growth was obserbed in two stages. The first growth began after the lag time in 14 hr and stopped for a while at 24 hr, but the second growth began after 70 hr and continued until after 160 hr when the turbidity of the culture medium reached about the same level as that of the maximum growth of control culture.

The existence of antimycin-resistant variants in culture medium was examined in the following way. One loopful of culture medium was taken at various culture times and streaked on glycerol medium containing 10^{-6} M antimycin in which antimycin-resistant cells could form colonies and sensitive cells could not.

As shown in Table I, we observed the existence of some antimycin-resistant variants when *C. utilis* was grown in glucose medium containing antimycin for more than 60 hr. The number of variants increased with the progress of culture time. But we did not observe the existence of any antimycin-resistant variant in the control culture at any culture time.

The emergence and the increase of antimycin-resistant variants population in glucose medium containing antimycin were investigated quantitatively as shown in Table II.

TABLE II. Variation of the Numbers of Viable Cells and of Antimycin-resistant Cells

Culture time (hr)	Number of viable cells (cells/ml)	Number of antimycin resistant cells (cells/ml)
48	$(1.28\pm0.18)\times10^{7}$	0
66	$(1.76\pm0.10)\times10^{7}$	0
72	$(1.08\pm0.12)\times10^{7}$	$(5.60\pm1.75)\times10^{4}$
96	$(4.95\pm0.45)\times10^{8}$	$(3.84\pm0.15)\times10^{8}$
144	$(4.08\pm0.15)\times10^{8}$	$(3.12\pm0.38)\times10^{8}$
168	$(2.20\pm1.18)\times10^{7}$	$(1.22\pm0.17)\times10^{7}$

Cells were inoculated into 100 ml of glucose medium (Sakaguchi's flask) containing 10^{-6} M antimycin to make a cell suspension of 10^{3} cells/ml and grown at 30° with reciprocal shaking. Culture media (1.0 ml) were taken, diluted adequately and spread on glucose medium plates to determine the number of viable cells and on glycerol medium plates containing 10^{-6} M antimycin to determine the number of antimycin-resistant cells. Figures in the Table show the cell number in the culture medium calculated from the numbers of colonies formed on each plate.

Antimycin-resistant variants could not be detected in the culture medium (0.1 ml containing about 10^{6} cells) taken at 48 hr or 66 hr. But in the culture medium grown for 72 hr the existence of antimycin-resistant variants was observed and the number of variants increased rapidly with an increase in each culture time.

The drug resistant cells of bacteria arise generally by spontaneous mutation and become predominant in the presence of drugs. We attempted to determine whether antimycin-resistant variants arose by spontaneous mutation.

Fluctuation Test

We carried out the fluctuation test by Luria and Delbruick. Cells were inoculated into fourteen L-shaped tubes containing 10 ml of glucose medium to make a cell suspension of 10^{3} cells/ml and grown for 72 hr. Both samples (about 4×10^{6} cells/0.1 ml) from independent

cultures and those from single culture were spread on glycerol medium containing 10^{-6} M antimycin and examined to see if antimycin-resistant variants existed.

As shown in Table III, we did not isolate any antimycin-resistant variant in the fourteen cultures, therefore we could not compare the deviation of variant numbers between independent cultures and single culture. We attempted further to compare the deviation under

Table III. Fluctuation Test on Emergence of Antimycin-resistant Variants

Sample No.	Numbers of antimycin-resistant variants			
	Cultured in glucose med. — Samples from		Cultured in glucose med. $+10^{-6}$ M antimycin — Samples from	
	Independent culture	Single culture	Independent culture	Single culture
1	0	0	9	178
2	0	0	35	190
3	0	0	94	144
4	0	0	75	175
5	0	0	143	163
6	0	0	177	140
7	0	0	95	96
8	0	0	82	120
9	0	0	9	158
10	0	0	30	138
11	0	—	16	—
12	0	—	416	—
13	0	—	0	—
14	0	—	178	—
Mean ± SD	0 ± 0	0 ± 0	84 ± 102	150 ± 22

Cells were inoculated into fourteen L-shaped tubes containing 10 ml of glucose medium with or without antimycin to make a cell suspension of 10^3 cells/ml and grown for 72 hr. Samples (0.1 ml of culture medium) from independent cultures or single culture were spread on glycerol medium containing 10^{-6} M antimycin and the number of colonies was counted after incubation for 72 hr at 30°

Table IV. Respiratory Activities of Antimycin-resistant Variants and Their Inhibition by Antimycin

Strain	Control	Antimycin		
		10^{-7} M	10^{-6} M	10^{-5} M
Parent	47.74	0.13	0	0
R-1	51.56	55.01	28.50	4.75
R-2	49.50	50.29	27.04	0.40
R-3	59.28	58.87	36.79	3.68
R-4	57.37	53.76	31.39	7.35
R-5	59.05	67.86	40.69	4.76
R-6	33.65	46.21	12.24	1.50
R-7	48.55	60.16	30.87	0.86
R-8	48.53	52.13	47.20	1.06
R-9	53.71	68.02	42.84	1.72
R-10	51.09	66.96	27.60	4.02

Values were expressed as nmoles of consumed O_2 per minute per mg dry weight. Cells were grown in Sakaguchi's flask containing 100 ml of glucose medium for 20 hours with reciprocal shaking. Respiration was measured conventional manometric procedures described under "Methods".

the condition where the variants arose. Cells were inoculated into glucose medium containing 10^{-6} M antimycin and grown for 72 hr. The existence of variants in each culture medium was examined as described above. In most of the cultures, the existence of variants was observed. The deviation of the number of variants in the samples of independent cultures was significantly greater than that of single culture as shown in Table III.

Effects of Antimycin on Respiratory Activities of Antimycin-resistant Variant

Several antimycin-resistant variants were isolated in each culture and named R-1—R-10. They were grown in glucose medium for 20 hr and their respiratory activities were measured in both the absence and the presence of antimycin.

The respiratory activities of variants (R-1—R-10) were about the same as the activity of parental strain. But some marked differences relating to the sensitivity to antimycin were observed between variants and parent. The respiration of variants was not inhibited by 10^{-7} M antimycin, while that of parental strain was remarkably inhibited. The respiration of variants was partially inhibited by 10^{-6} M antimycin though the sensitivity to antimycin was different in each variant. When the concentration of antimycin rose to 10^{-5} M, a remarkable inhibition of respiration was observed in all variants.

Discussion

Several antimycin-resistant mutants of *C. utilis* have been isolated by treatment with mutagen. Butow *et al.*[4] isolated antimycin-resistant mutants by treatment with acriflavine and Grimmelikhuizen *et al.*[5] isolated by treatment with sodium nitrite.

But we found a convenient method of isolating antimycin-resistant cells without treatment with mutagen. Since original cells were all sensitive to antimycin, they did not grow in glycerol medium containing antimycin (Fig. 1, Table I and Table II), and two-stage growth was observed when they were grown in glucose medium containing antimycin (Fig. 2).

From these results, it is formulated that all inoculated cells were sensitive to antimycin and that they grew by fermentation until glucose was exhausted out (the first growth). In this process antimycin-resistant cells arose and grew exclusively by utilizing the fermentation product of glucose (the second growth).

All antimycin-resistant variants isolated in each independent culture grew uniformly in a medium containing 10^{-5} M antimycin (the related data not shown). The resistance of these variants to antimycin remained stable after they were subcultured repeatedly in glucose medium without antimycin (the related data not shown).

As shown in Table III, results obtained from fluctuation test suggest that antimycin-resistant variants arise unperiodically by spontaneous mutation. In order to verify this assumption, it seems to be necessary to isolate some antimycin-resistant variants under the condition where the cells do not contact with antimycin.

The respiratory activities of these antimycin-resistant variants were on the same level as the activity of parental strain, though the sensitivity of respiration to antimycin was different between variants and parent. The respiration of variants was not inhibited by 10^{-7} M antimycin but inhibited by the higher concentrations of antimycin. Therefore these variants do not seem to become antimycin-resistant by the possession of any special antimycin-resistant respiratory chain as reported about *Eugrena gracilis*,[6] *Ustilago maydis*,[7] *Neurospolla sitophila*[7] or *Aspergillus oryzae*.[8] But other possibilities such as a decrease in

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6) T.K. Sharpless and R.A. Butow, *J. Biol. Chem.*, **245**, 58 (1970).
7) J.L. Sherald and H.D. Sisler, *Plant physiol.*, **46**, 180 (1970).
8) M. Kawakita, *J. Biochem.* (Tokyo), **69**, 35 (1971).

membrane permeability of antimycin, a reduction in affinity to the electron transport particle of antimycin or an increase in enzyme activity that inactivates antimycin should be considered. The sensitivity of isolated mitochondria to antimycin is now under investigation and will report elswhere.[9]

Acknowledgement We are grateful to Dr. M. Yoshikawa of the institute of medical science, university of Tokyo for his helpful suggestions about fluctuation test, to Mr. K. Kakihara and Miss Y. Honda for their skilled technical assistances.

9) Y. Iwamoto and I. Mifuchi, in preparation.