ANTIMYCIN RESISTANCE IN SACCHAROMYCES CEREVISIAE : A NEW MUTATION ON THE mitDNA CONFERRING ANTIMYCIN RESISTANCE ON THE MITOCHONDRIAL RESPIRATORY CHAIN

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SUMMARY: Mutants of S. cer. were selected, which are resistant to antimycin (inhibitor of electron transport in the bc_1-segment of the respiratory chain). The mutant A^R1o1 is described in detail. 1.) The mutation conferring antimycin-resistance is located on the mitochondrial DNA (mitDNA). In recombination analysis, A^R1o1 is not linked to the known markers C, E, O_I and P. 2.) The in-vitro resistance of the NADH oxidase can only be demonstrated by using competitors for antimycin: ubiquinone-3 (Q-3) or bovine serum albumine (BSA) (see B.Lang et al. (1975) Molec. gen. Genet. 137 , 353). Q-3 increases the antimycin tolerance of the NADH oxidase activity in mutant but not in wild-type mitochondrial particles. Mitochondrial respiration inhibited by antimycin is restored by BSA in A^R1o1 only.

INTRODUCTION:

Antimycin is the most potent inhibitor known for mitochondrial respiration and has been studied very intensively for a long time. Yet, the reactions of antimycin are not fully understood, obviously due to a number of contradictory theories regarding its inhibition in the bc_1-segment of the respiratory chain (1-7). Moreover, little is known about the function, structure and biogenesis of cytochrome b (8) and its uncertain identity with an antimycin binding-site (3,9).

The great value of antimycin-resistant mutants for the biochemical analysis of antimycin inhibition and mitochondrial genetics in Schizosaccharomyces pombe has already been shown (1,6,10,11). However, in S. cer. this type of mutants has not yet been described.

In this communication we describe the isolation of antimycin-resistant mutants in S. cer. . It is shown that the mutation A^R101 confers antimycin-resistance to the respiratory chain and is mitochondrially inherited.

MATERIALS AND METHODS

a.)Strains : SM 11-6C (12); SM 11-6C/1o1 = A^R1o1 ; IL 993-5C (13); BM 1421-9C (14); SM 821-7C (12); KL 58-2A (13).

b.)Abbreviations : Symbols of genetic markers and the nomenclature correspond to (12,13). A^R/A^S are allelic forms of a locus conferring resistance/sensitivity to antimycin.
SDS, sodium dodecyl sulfate; BSA, bovine serum albumine; FCCP, carbonyl cyanide-p-trifluormethoxyphenylhydrazone; NG, N-methyl-N'-nitro-N-nitrosoguanidin; Q-3, ubiquinone-3.

c.)Media : The media YPG, YPGD, YPG-C, YPG-E, YPG-O and YPG-P are described elsewhere (12,15). YPG-A plates were obtained by plating an ethanolic solution of antimycin on YPG agar to a final concentration of 5 nM.

d.)Chemicals : Erythromycin and horse-heart cytochrome c was purchased from Boehringer, Mannheim; oligomycin from Serva, Heidelberg; paromomycin from Parke Davis, Munich; antimycin from Nutritional Biochemical Corporation and Urografin from Schering, Berlin. Chloramphenicol was a gift from Bayer, Leverkusen; Q-3 from Dr. Solms and FCCP from Dr. Heytler.

e.)Isolation of mutants : Mutants resistant to antimycin were obtained in strain SM 11-6C by the following procedure: After treating different batches of 5 x 10^8 cells with NG (survivals 10, 20, 50 %), the cells were washed, then resuspended in 100 ml liquid YPGD medium (3% glycerol and 0.1% glucose), and 0.5 µM antimycin and 20 µg/ml SDS (increasing cell permeability (16)) added. Cultures were shaken at 30°C for three days, leading to a titer of 5 x 10^7 - 1 x 10^8 cells per ml. Then aliquots were plated on selective media (YPG-A) and incubated at 30°C. A^R mutants arising within 3 days were isolated. In control experiments, when NG treatment of the cells was omitted, no A^R mutants were detected.

f.)Genetic analysis of multifactorial mitochondrial crosses : The quantitative random analysis of the progeny of zygote populations is described elsewhere (12).

g.)Preparation of mitochondria : Well aerated, glycerol-grown cells were harvested from liquid medium in the late logarithmic phase at a titer of 2 x 10^8 cells per ml and broken by a mechanical procedure described elsewhere (17). Mitochondria were isolated after centrifugation for 20 min at 46000 x g and further purified by flotation in a linear Urografin gradient (40-20 %)(60 min at 136000 x g in a Spinco SW 27 swing-out rotor). The mitochondrial fraction was diluted by addition of 0.6 M sorbitol , 10 mM EDTA and centrifuged for 20 min at 46000 x g. Mitochondrial particles were stored under liquid nitrogen up to 4 weeks.

h.)Enzymatic analysis : Since the cytochrome b content of mitochondrial preparations from wild-type and mutant of S. cer. obtained by this procedure was found to be between 450 - 540 pmol/mg protein (an absorbance coefficient of $\varepsilon_{563-573} = 26\ mM^{-1} \times cm^{-1}$ was assumed), protein determinations were carried out via cytochrome b determination based on the average of 500 pmol cytochrome b per mg protein.
The cellular respiration was tested in liquid YPGD medium, the mitochondrial respiration in a buffer containing 80 mM KH_2PO_4, 25 mM Tris, 1 mM EDTA and 0.1 mM cytochrome c, pH 7.4 with a Clark type electrode at 25°C. FCCP, inhibitor or ubiquinone were added in ethanolic solution, not exceeding a final concentration of 1 % ethanol.

RESULTS

The measurement of cell-growth as a function of the antimycin concentration was performed in a pair of isonuclear diploid strains (mitotic segregants of cross No. 1 in TABLE 1A), one carrying mutation A^R101, the

TABLE 1: Result of four multifactorial crosses involving the alleles A^R and A^S.

A

cross No.			Transmission (%) C^R	E^S	O^S	P^S	A^R	colonies
1.)	SM 11-6C $\omega^- C^R E^S O^S P^S A^R$	x IL 993-5C $- C^S E^R O^R P^R A^S$	35.5	35.2	32.2	33.6	41.0	369
2.)	SM 11-6C $\omega^- C^R E^S O^S P^S A^R$	x BM1421-9C $- C^R E^S O^R P^R A^S$	–	–	54.2	54.2	54.4	535
3.)	SM 11-6C $\omega^- C^R E^S O^S P^S A^R$	x SM 821-7C $- C^S E^R O^R P^R A^S$	–	58.3	60.1	–	52.9	582
4.)	SM 11-6C $\omega^- C^R E^S O^S P^S A^R$	x KL 58-2A $- C^R E^R O^S P^R A^S$	–	68.2	–	74.6	67.6	485

B

cross No.	Recombination (%) $C^R E^R$ / $C^S E^S$	$C^R O^R$ / $C^S O^S$	$C^R P^R$ / $C^S P^S$	$E^S O^R$ / $E^R O^S$	$E^S P^R$ / $E^R P^S$	$O^S P^R$ / $O^R P^S$	$C^R A^S$ / $C^S A^R$	$E^S A^S$ / $E^R A^R$	$C^S A^S$ / $C^R A^R$	$P^S A^S$ / $P^R A^R$
1.)	5.4 / 5.7	10.6 / 7.9	10.3 / 8.9	9.5 / 6.5	10.6 / 8.9	7.0 / 8.4	7.6 / 13.6	8.7 / 14.4	6.8 / 14.1	3.8 / 12.5
2.)	–	–	–	–	–	13.6 / 13.5	–	–	12.5 / 12.7	11.0 / 11.4
3.)	–	–	–	9.8 / 11.7	–	–	–	11.7 / 6.4	15.5 / 8.2	–
4.)	–	–	–	–	5.6 / 12.0	–	–	9.7 / 8.7	–	13.0 / 5.6
Recomb. colonies	41	68	71	184	157	202	78	279	350	270
Average%	11.1	18.4	19.2	19.3	18.4	22.3	21.1	19.4	23.6	19.4

The progeny of zygote populations was analyzed by replicaplating colonies of essentially homoplasmic diploid cells on media discriminating between C^R and C^S (4 mg chloramphenicol/ml YPG), E^R and E^S (2 mg erythromycin per ml YPG), O^R and O^S (3 µg oligomycin/ml YPG), P^R and P^S (2 mg paromomycin/ml YPG) or A^R and A^S (o.o1 µg antimycin/ml YPG). Crosses and analyses of phenotypes were performed at 30°C.
Alleles used correspond to the following loci: RIB1 (C^R321), RIB3 (E^R221) OLI1 (O^R1) and PAR1 (P^R454).
A: Transmission (%) refers to the frequencies of colonies carrying the respective allele; B: Recombination (%) refers to the frequencies of recombinant colonies among the progeny of a cross. The frequency of each of the two possible reciprocal recombinants is given.

other the wild-type allele A^S. In fermentable substrate (dextrose) inhibition of both strains is observed only in the presence of more than 5 nM and a 50% inhibition achieved by a concentration of 500 nM antimycin. In non-fermentable substrate (glycerol) 50% inhibition is obtained by 1.2 nM antimycin in strain A^S and by 14 nM in A^R101. A concentration of 5 nM antimycin allows excellent discrimination of growth between A^R and A^S cells, both in liquid and on solid YPG medium. Resistance of cell growth of A^R101 against antimycin corresponds with resistance of cellular respiration (concentration necessary for a 50% inhibition is about 11 times higher in A^R101 than for the wild-type, Fig.1). Uncoupling of cellular respiration by FCCP (15 /uM) increases the oxygen-consumption in both strains (stimulation factor between 2.2 - 2.7) and is not affected by antimycin concentrations causing less than 70% inhibition. A phenomenon only observed in the mutant was the increased sensitivity to antimycin of the cellular respiration, when cells were preincubated under anaerobic conditions in the presence of antimycin. 20 min preincubation of cells with an antimycin concentration leading to 50% inhibition in non-preincubated cells causes an increased inhibition (95%) followed by a slow restoration of activity during 5-15 min of electron flow.

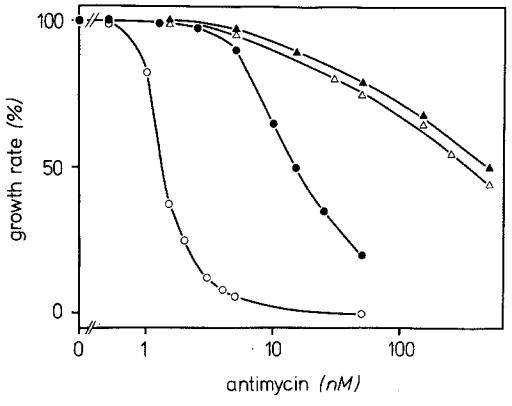

Figure 1: Inhibition of exponential growth of A^R and A^S cells as a function of antimycin concentration

Isonuclear diploid strains carrying the mutant allele A^R101 or the corresponding wild-type allele A^S were grown in liquid YP medium (30°C) containing dextrose (fermentable substrate) or glycerol (non-fermentable substrate) and various concentrations of antimycin (abscissa). For each concentration the growth rate during the exponential growth phase was determined by optical density measurement.
A^R, grown in dextrose(▲—▲);A^S, grown in dextrose (△—△);
A^R, grown in glycerol(●—●);A^S, grown in glycerol (○—○).

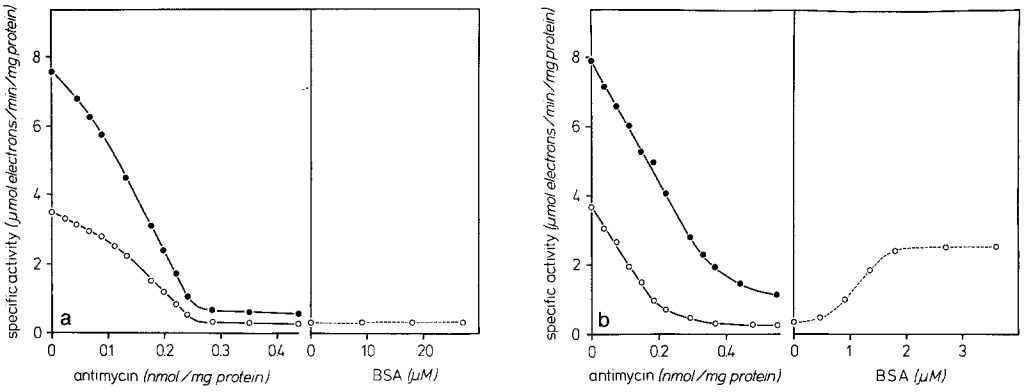

Figure 2A: Inhibition of the NADH oxidase by antimycin in the wild-type (O — O), effect of ubiquinone-3 on the inhibition of the NADH oxidase (● — ●) and restoration of the inhibited (90%) activity by BSA (O---- O).
The concentration of protein was 42 µg/ml, of Q-3 0.1 mM.

Figure 2B: Inhibition of the NADH oxidase by antimycin in the mutant A^R101 (O — O), effect of ubiquinone-3 on the inhibition of the NADH oxidase (● — ●) and restoration of the inhibited (90%) activity by BSA (O---- O).
The concentration of protein was 38 µg/ml, of Q-3 0.1 mM.

The concentration of antimycin causing 90% inhibition of the NADH oxidase in isolated mitochondrial particles was equal in mutant and wild-type. Nevertheless, an alteration of the respiratory chain, caused by mutation A^R101 could be demonstrated by the following two experiments:
-BSA, which binds antimycin (3), was added to antimycin-inhibited mitochondrial particles. This resulted in the restoration of NADH oxidase activity in mutant A^R101 but not in wild-type (Fig.2).
-A similar effect was observed after adding Q-3 (1,5,17,19), which acts like the natural Q-6. It increases the NADH oxidase activity both in mutant and wild-type mitochondria. However, in the mutant the concentration of antimycin necessary for a 50% inhibition of the stimulated activity is significantly higher than for the unstimulated activity (0.23 and 0.12 nmol/mg protein, respectively). The NADH oxidase-inhibition curves varied between linear and slightly sigmoidal. Curves, representative for the two strains are shown in Fig.2. From a series of experiments it seems that sigmoidicity of the curves and low respiratory activities correspond. However, sigmoidicity is in general stronger in wild-type. It must be emphasized that full response of mitochondrial

respiration upon addition of Q-3 or antimycin was observed only after more than 5 min of electron flow. Measurements that were made before maximal response was reached resulted in clearly sigmoidal inhibition curves.

Four crosses (rho^+ x rho^+) were performed involving mutation A^R101 and the mitochondrial mutations C^R321, E^R221, O^R1 and P^R454 which confer resistance against chloramphenicol, erythromycin, oligomycin and paromomycin, respectively (for review see 20). The progeny of zygote populations of each cross was analyzed and two parameters were calculated from the result of these crosses, A: transmission of markers and B: recombination between markers (TABLE 1). Resistant and sensitive alleles of each gene segregate frequently among the progeny of each cross. Transmission of A^R is covariant with other markers (TABLE 1A), e.g. high (low) frequency of transmission of the allele A^R in a cross is accompanied by high (low) frequencies of the other alleles derived from the A^R parental strain. The frequency of recombinants between any pair of markers, including or not including A^R, does not exceed 25% (TABLE 1B). These results are consistent with the model of mitochondrial recombination (21) predicting covariance of transmission and an upper limit of recombination between mitochondrial markers which are not affected by alleles causing polarity of recombination.

Recombinant frequencies between A^R101 and any of the other markers analyzed in these crosses are likewise close to 20%, a result also found with other pairs of markers not including A^R101, except the pair C-E. This indicates no linkage between A^R101 and the other markers analyzed.

DISCUSSION

According to the currently accepted criteria for mitochondrial inheritance A^R101 is a mitochondrial mutation site as has been shown previously for the markers C^R321, E^R221, O^R1 and P^R454 (for review see 20). The absence of apparent linkage to any of these previously studied mitochondrial resistance markers signifies that mutation A^R101 refers to a new mitochondrial locus which confers resistance/sensitivity to antimycin, an antibiotic previously not used in mitochondrial genetics of S. cer. . This fact and the good discrimination of A^R and A^S cells as well as the lack of cross-resistance to other antibiotics used in mitochondrial genetics make the marker A^R1o1 useful for further recombination studies.

The data presented reveal good evidence that mutation A^R1o1 causes functional changes in the respiratory chain. Although resistance of cell

respiration to antimycin of the mutant is high, in-vitro expression of the mutation in mitochondrial components could be shown only indirectly: Inhibition of the NADH oxidase by antimycin - which is the same in mitochondrial particles of mutant and wild-type - is reduced only in the A^R1o1 strains if Q-3 or BSA is present. In beef-heart comparable regulatory effects of ubiquinone on the action of antimycin have been reported (19).

With respect to the inheritance and the phenotypic expression of the described mutation, A^R1o1 is similar to an antimycin resistant mutant of Sch. pombe, ANT^R8 (1,1o,11). Only two differences in the phenotypic expression have been found so far : -in mutants of Sch. pombe the NADH oxidase of mitochondrial particles is highly resistant to antimycin, - A^R1o1 is cross-resistant to HQNO in contrast to ANT^R8 (6, 22).

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