YEAST MUTANTS RESISTANT TO BONGKREKIC ACID, AN INHIBITOR OF MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCATION

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1. Introduction

The genetic modification of resistance to inhibitors of oxidative phosphorylation was introduced by Parker et al. [1] with the isolation of oligomycin-resistant yeast mutants. Subsequently some *Saccharomyces cerevisiae* mutants have been found which are resistant to uncouplers, others resistant to triethyl tin [2], and very recently *Kluyveromyces lactis* mutants resistant to alkylguanidines have been isolated [3]. Such mutants are valuable for studying both mitochondrial function and biogenesis [4, 5]. This communication describes a new type of *S. cerevisiae* mutant which is resistant to an inhibitor of mitochondrial adenine nucleotide translocation, bongkrekic acid (BA) [6, 7]. In tetrads derived from crosses between normal and resistant strains segregation is 2 resistant: 2 sensitive, indicating that resistance is determined by a single nuclear gene, *bka*1.

2. Materials and methods

BA was prepared from *Pseudomonas cocovenenans* (strain NCI B 9450) by the method of Nugteren and Berends [8] modified by Lijmbach et al. [9], except that the last step involving a purification by countercurrent distribution was replaced by a chromatography on a DEAE-Sephadex column. BA separated from other components by thin layer chromatography on silica gel was eluted by methanol as described in [9]; the methanolic solution was concentrated and mixed with a 20 mM morpholinopropane sulfonate (MOPS) buffer, pH 7.5. Between 5 and 10 mg of BA in 1 ml of methanol were added to 9 ml of 20 mM MOPS buffer, pH 7.5 and applied to the top of a DEAE-Sephadex column (20 cm × 2 cm) previously equilibrated with 20 mM MOPS, pH 7.5, 0.1 M NaCl. After the column had been washed with 50 ml of the equilibration buffer, the elution of BA was carried out with a linear NaCl gradient ranging from 0.1 M–1 M in 20 mM MOPS buffer, pH 7.5. The eluates were monitored with an Uvicord Cell at 253 nm and collected with an LKB fraction collector. After acidification with diluted sulfuric acid to pH 2, BA was extracted in diethylether (peroxide free) and transferred after diethylether concentration into 2 N ammonia. The final concentration of the ammonia salt of BA was calculated from the value of $\epsilon_{263} = 40.6\ cm^{-1} \times mM^{-1}$.

In order to conserve the limited amount of BA available, a semi-microsystem for producing mutants, and for resistance assay was devised. This involved use of small Petri dishes containing 3 ml of YPAE medium containing 1% yeast extract, 2% peptone, 3% (v/v) ethanol, 20 mg adenine sulfate/liter and 1% Ionagar #2 (Colab); pH was 6.2. To prevent excessive evaporation, plates were incubated at 30°C in a tightly covered plastic box containing a moist paper towel. Assay plates containing BA were prepared by first applying 0.1 ml of sterile, aqueous Tween 80 (50 mg/ml)

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Table 1
Resistance of yeast mutants to different BA concentrations.

BA concentration (mM)	No. of strains resistant
0.15	17
0.20	15
0.30	0

Mutants were derived from strain GT34-6A after NMG treatment. Resistance was determined by estimating growth on YPAE+BA at 30°C. Growth was scored daily for 4 days.

and an appropriate small volume of concentrated BA solution to the agar surface, then spreading the mixture with a bent glass rod. Up to 16 test spots per plate may be applied with a 1 mm inoculating loop using suspensions of about 10^6 cells/ml.

Inbred *S. cerevisiae* strains GT34-6A (a *his*1, *lys*2, *trp*2) and GT34-1B (α *adel*) were selected for efficient growth on YPAE and for similarity in sensitivity to BA. Mutants were derived from GT34-6A by treatment with *N*-methyl-*N'*-nitrosoguanidine (NMG) as previously described [10]. Mutagenized cells were plated on YPAE containing 0.15 or 0.3 mM BA and the resistant clones selected were purified by replating on YPAE+0.15 mM BA. Mutant H63 and parental strain GT34-6A were crossed with GT34-1B, and tetrad analyses were performed by the method of Hawthorne and Mortimer [11].

3. Results

3.1. *Isolation of mutants*

Two difficulties were encountered in first attempt to isolate suitable BA-resistant mutants. First, mutants with enhanced efficiency for ethanol utilization were found. Second, when the first two complementary, BA-sensitive strains chosen were crossed and allowed to sporulate, a large number of BA-resistant segregants were produced. This suggests that these particular parental strains contain certain genes which singly do not cause resistance, but when properly recombined in new progeny bestow considerable BA resistance. Therefore, to decrease the chances of such interference, new strains were selected from among the BA-*sensitive* progeny and used for subsequent work.

Table 2
Inheritance of resistance to bongkrekic acid.

	No. of tetrads of type (R:S)				
Diploid no.	0:4	1:3	2:2	3:1	Totals
GT35	0	1	15	1	17
GT36	12*	3	0	0	15

* Two tetrads contained one very weakly resistant segrant each. R-resistant, S-sensitive. Diploid GT36 was derived from the two sensitive parental strains. Resistance was determined as in table 1, using 0.15 mM BA. Nutritional markers segregated 2:2.

One of these new strains, GT34-6A, was treated with NMG, and 17 mutants were selected. As shown in table 1 all mutants were resistant to 0.15 mM BA, most to 0.20 mM, but none to 0.3 mM. One of the more rapidly growing resistant mutants, H63, was selected for genetic analysis.

3.2. *Inheritance of BA resistance in strain H63*

Both mutant H63 and the sensitive parent GT34-6A were crossed with sensitive tester strain GT34-1B to give the respective diploids, GT35 and GT36 (control). These diploids were allowed to sporulate and tetrad analyses were performed. Table 2 presents the results of the analysis of BA resistance segregation. Since the great majority of tetrads from GT35 contained 2 resistant segregants, and since in the control, GT36, resistant recombinants were rare, it is concluded that the BA resistance in H63 is determined by a single, nuclear gene, *bka*1. This gene appears to be recessive, since growth of the heterozygous diploid, GT35, is inhibited by 0.15 mM BA.

4. Discussion

The successful isolation of BA-resistant yeast mutants is of potential utility in studying the mechanism of mitochondrial adenine nucleotide translocation, and may provide information about this process not readily obtained by other means [6, 7, 12, 13]. For example, mitochondria isolated from BA-resistant mutants could be used to test whether the site of BA action is identical with the site of actractyloside action.

Although resistance to BA may conceivably arise by changes in cell membrane or by an enhancement of a detoxication process, it is likely that among the mutants isolated, many will prove to have an altered mitochondrial membrane. Identification of mutants of the latter class, made by studying BA effects on isolated mitochondria, may be followed by genetic complementation studies [10] to estimate how many polypeptides are associated with BA-sensitive translocation. The existence of polygenic control is suggested by our preliminary studies which showed that BA-resistant meiotic progeny could be derived from crosses between certain types of BA-sensitive haploid parents.

If it can be shown subsequently that *bka*1 affects a mitochondrial protein directly, then the Mendelian inheritance of this gene would indicate that biogenesis of at least a portion of the adenine nucleotide translocation system is not determined directly by mitochondrial DNA. However, genetic analysis of the other BA-resistant mutants described in this work may subsequently implicate mitochondrial DNA.

The antifungal activity of BA has previously been described. *Pseudomonas cocovenenans*, the organism which produces BA, is ecologically associated with the phycomycete *Rhizopus oryzae* which is used in preparation of the Indonesian coconut product, *bongkrek*. BA was shown to be a potent inhibitor not only of *Rhizopus* but also of yeast and *Penicillium glaucum* [14]. Since intact yeast cells are susceptible to BA inhibition, it should be possible to utilize BA for *in vivo* studies of mitochondrial function utilizing approaches such as those developed by Chance [15] and by Maitra and Estabrook [16]. In fact, an *in vivo* study with yeast has been performed by van Veen, who showed that BA-treated yeast cells were deficient in glycogen, as indicated by iodine staining (Lugol's solution).

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