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Mitochondrial mutations affecting resistance to erythromycin and mikamycin in *Paramecium aurelia:* provisional results with a new method

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SUMMARY

A new method for obtaining mitochondrial mutants of *Paramecium aurelia* is described. Sensitive organisms were first placed in medium containing a low concentration (0·02 mg/ml) of erythromycin (ERY), insufficient to prevent fission. The paramecia were then transferred through a graded series of increasing ERY concentrations, with a period of growth at each concentration. A high yield of ERY-resistant mutants was obtained.

Continuous growth in 0·02 mg/ml ERY also resulted in the accumulation of a spectrum of mutants resistant to various concentrations of ERY. Many of these mutants are also resistant to mikamycin. On returning the resistant paramecia to media lacking ERY, some clones remained resistant whilst others reverted to sensitivity.

1. INTRODUCTION

Very little is known about mutation of mitochondrial genes. Following earlier work with yeast (Coen *et al.* 1970; Linnane *et al.* 1968; Thomas & Wilkie, 1968), it was shown that in *Paramecium* the change from erythromycin-sensitivity to resistance is due to mitochondrial mutations (Beale, Knowles & Tait, 1972), but exact data on mutation frequency and on the conditions for selection and expression of mutants are lacking. The present paper is a preliminary account of studies using a new method to obtain information on these questions.

The following abbreviations will be used: erythromycin – ERY; mikamycin – MIK; ERY-resistant mutants – E^R; ERY-sensitives – E^S; MIK-resistants – M^R; MIK-sensitives – M^S.

In previous work (Beale, 1969; Adoutte & Beisson, 1970), E^R mutants were obtained by suspending wild-type (E^S) paramecia in media containing 0·1 mg/ml or 0·25 mg/ml ERY, which inhibits growth of E^S cells, (apart from one or two residual fissions) without, however, killing the paramecia. After a period of 2–4 weeks, such suspensions usually produce some actively growing paramecia, which, on isolation, can be shown to be E^R mutants. Approximately $1/10^3$ originally isolated paramecia yielded E^R mutants by this method, and on the very rough assumption that there are 5000 mitochondria per paramecium, this would indicate a mutation frequency of $1/10^6$ to $1/10^7$ mitochondria. However, at least two classes of E^R

mutant were produced by selection in 0·1 mg/ml ERY; one resistant to 0·1 mg/ml ERY and also to 0·225 mg/ml MIK, the other resistant to 0·25 mg/ml ERY but sensitive to MIK (Beale, 1973). Probably other classes of mutant resistant to different concentrations of ERY and cross-resistant to other antibiotics also occur.

2. SELECTION OF MUTANTS IN A GRADED SERIES OF ERY CONCENTRATIONS

As already mentioned, in the method previously used, mutants arose, or were phenotypically expressed, in a population of *non-dividing* cells, which were exposed to a high concentration of ERY. A similar situation probably existed in the experiments with yeast (Birky, 1973). A more abundant yield of mutants might be expected to be obtained amongst a population of dividing cells, and one exposed to a lower concentration of ERY, which would have a weaker inhibitory action on the process involved in the expression of new mutations. Accordingly, in the experiments now to be reported, a sample of 24 E^S paramecia (*P. aurelia*, stock 513, syngen 1) were isolated in depression slides containing bacterized medium with 0·02 mg/ml ERY, which permits fission, though at a reduced rate.

After about seven fissions and a subsequent period without fission of about 14 days samples of six animals from each of seven clones (chosen at random from the survivors) were transferred to medium containing 0·05 mg/ml ERY, again allowed to pass through about seven fissions, and then transferred by stages to media containing 0·10 mg/ml, 0·125 mg/ml and finally 0·25 mg/ml ERY. At all stages, samples were tested for ability to grow in all the ERY concentrations (Table 1).

Table 1. *Transfer of paramecia through increasing concentrations of ERY*

ERY concentration (mg/ml)	No. of paramecia initially placed in medium	Approx. number of fissions undergone	Number of resistant clones obtained
0·02	24*	7	15
0·05	42**	7	12
0·10	72†	7–17	31
0·125	186‡	7–17	109
0·25	109§	7	88

* Each of these 24 paramecia initiated the development of a clone.

** These 42 paramecia were obtained by taking six animals from each of seven clones (out of 15) surviving the 0·02 mg/ml ERY treatment.

† These 72 were obtained by taking six animals from each of the twelve clones surviving the 0·05 mg/ml ERY treatment.

‡ These 186 were obtained by taking six animals from each of the 31 clones surviving the 0·10 mg/ml ERY treatment.

§ These 109 were obtained by taking one animal from each of the 109 clones surviving the 0·125 mg/ml ERY treatment.

The results may be summarized as follows. (1) Wild-type (E^S) paramecia failed to grow in any ERY concentration higher than 0·02 mg/ml. (2) After growth in a given ERY concentration, a high proportion (20–80%) of the survivors acquired

the ability to grow in the next higher concentration, and in a few cases in the highest (0·25 mg/ml). (3) One such highly resistant E^R mutant, was tested by crossing with E^S cells, and shown to have developed a permanent, cytoplasmically inherited ERY resistance. (4) While it is impossible to estimate the 'mutation frequency' from these experiments, it is evident that a high proportion of the cells initially isolated eventually yielded E^R mutants by this method.

3. CROSS-RESISTANCE OF E^R MUTANTS TO MIK

Since in earlier work (Beale, 1973) some E^R mutants were found to be resistant also to MIK, it was of interest to determine at what stage such M^R paramecia might appear in experiments involving exposure of paramecia to a low concentration (0·02 mg/ml) of ERY.

To investigate this, 36 E^S paramecia were isolated in medium containing 0·02 mg/ml ERY and transferred three times a week to fresh medium containing the same ERY concentration, for about 9 weeks, keeping the cultures at 25 °C. About once a week, paramecia from all 36 clones (excluding of course those dying) were tested for ability to grow in two media, one containing 0·10 mg/ml ERY and the other 0·225 mg/ml MIK. The results are given in Table 2 and rather surprisingly show that resistance to MIK (i.e. ability to undergo fission in presence of the antibiotic) seemingly appears sooner, and at a higher frequency, than resistance to ERY. Thus, by the 22nd day 6 out of 33 clones were able to grow in 0·225 mg/ml MIK but none in 0·1 mg/ml ERY, and on the 28th day 10 out of 31 clones grew in 0·225 mg/ml MIK and only 5 out of 31 grew in 0·1 mg/ml ERY.

Table 2. *Acquisition of resistance to ERY and MIK following growth of clones from 36 paramecia in medium containing 0·02 mg/ml ERY*

No. of days paramecia grown in 0·02 mg/ml ERY	No. of fissions in 0·02 mg/ml ERY	No. of clones surviving in 0·02 mg/ml ERY	No. of clones resistant to 0·1 mg/ml ERY	No. of clones resistant to 0·225 mg/ml MIK
0	0	36	0	0
2	7	36	0	0
22	42	33	0	6
28	52	31	5	10
34	67	29	8	17
41	82	29	12	26
49	97	29	11	26
57	112	28	11	25
64	127	28	11	25

A similar experiment was carried out using 0·045 mg/ml MIK (in place of 0·02 mg/ml ERY) as the initial selecting medium. The results showed that MIK (at least at the concentration used) is less efficient than ERY in promoting the origin and/or survival of mutants, even those resistant to MIK itself. After 57 days on 0·045 mg/ml MIK, only 7 clones out of 35 had become resistant to 0·225 mg/ml MIK and none were resistant to 0·10 mg/ml ERY.

4. STABILITY OF THE RESISTANT CLONES

Paramecia from six E^R clones obtained in the experiment depicted in Table 2 were transferred back to medium lacking antibiotics, and tested for reversion to ERY and MIK sensitivity, after varying times. The results are given in Table 3. They show that some clones (nos 22 and 30) lost resistance to both antibiotics, others (nos. 29 and 36) showed a partial loss of resistance to one or other antibiotic, whilst two clones (nos. 25 and 49) showed no loss of resistance under these conditions.

Table 3. *Reversion from resistance to sensitivity following growth of resistant paramecia in non-selective medium*

Clone no.	Days in medium containing 0·02 mg/ml ERY	Days subsequently in non-selective medium	Resistance in: 0·05 mg/ml ERY	0·10 mg/ml ERY	0·225 mg/ml MIK	0·45 mg/ml MIK
22	57	0	+	−	+	+
	64	28	−	−	−	−
30	64	0	+	−	+	−
	92	0	+	+	+	+
	64	28	−	−	−	−
29	57	0	+	−	+	−
	64	28	+	−	−	−
36	28	0	+	+	+	
	57	0	+	+	+	+
	64	28	+	−	+	+
25	57	0	+	−	−	−
	64	28	+	−	−	−
49	28	0	+	+	+	Not tested
	57	0	+	+	+	+
	64	28	+	+	+	+

5. DISCUSSION

The results described above show that by exposing paramecia to a low, non-fission-inhibiting concentration of ERY, and raising the concentration in a stepwise manner, a high proportion of E^R mutants is obtained, much higher than by the earlier method of immediate exposure to a high concentration of ERY. By the new method, a spectrum of ERY resistant and MIK cross-resistant mutants is obtained. Moreover, continuous exposure to the low concentration of ERY results in a steady increase in number of mutants resistant both to ERY and to MIK. Some of the ERY results appear to be stable on returning the paramecia to medium lacking antibiotic; others are unstable under these conditions, apparently reverting to sensitivity through a series of stages.

By way of explanation of these results, we assume that the development of the resistant phenotype requires three main events. First, the mitochondrial DNA in

individual mitochondria must change in such a way as to give rise to a mutation. Secondly, the potential mutant mitochondrion must change in such a way as to exhibit the mutant phenotype, to function metabolically and replicate in presence of the antibiotic. Thirdly, the newly resistant mitochondrion has to replicate and displace the pre-existing sensitive mitochondria in a cell. Only when a substantial majority of the mitochondria in a cell are resistant, is the cell itself able to grow in presence of antibiotic (Knowles, 1972). Thus, appearance of a resistant mutant paramecium requires the occurrence of a primary mutational event followed by its expression at two levels, that of the individual mitochondrion and that of the whole cell. Moreover, there are mutants resistant to different concentrations of antibiotic, and there may be a succession of mutational events, each adding to the resistance.

In view of the complexity of these changes, the term 'mutation frequency' is somewhat meaningless in this situation. The primary mutational change in these experiments is presumed to occur spontaneously (there is no evidence whether ERY or MIK can act as mutagens) but the second and third stages are likely to be affected by the presence of antibiotic in the medium. These substances are known to inhibit protein synthesis on mitochondrial ribosomes. If there is a high concentration of antibiotic in the medium, protein synthesis may be inhibited and the expression of a mutation prevented. If there is no antibiotic present at all, such spontaneous mutations as occur may fail to be revealed. Even though the mutant mitochondrial phenotype might develop, selection at the expense of non-mutants may not take place, since it is likely that mutant mitochondria are at a disadvantage in competition with non-mutants in non-selective media. (This has been demonstrated by mixing two kinds of mitochondria in the cytoplasm of individual paramecia (Beale, Knowles & Tait, 1972; Adoutte & Beisson, 1972).)

Thus, the most favourable condition for development and retention of resistant mutants would be a low initial concentration of ERY. As partial resistance develops, a higher concentration can be tolerated and further mutants allowed to accumulate.

The 'instability' of resistance in some clones when the paramecia are returned to non-selective media, may be interpreted on the assumption that a mutant cell phenotype may develop before all sensitive mitochondria in a given cell have been eliminated. This seems particularly likely where low concentrations of antibiotic are used, insufficient to suppress replication of non-mutant mitochondria, and sufficient only to place them at a slight disadvantage to the mutants. When such 'heterogeneous' cells, containing both mutant and non-mutant mitochondria, are placed in non-selective media, the non-mutants would once again become predominant and the cells lose resistance.

Another possibility is back-mutation (from resistance to sensitivity), but this would seem less likely since previous work has shown that, once stable mutants are obtained, they rarely, if ever, show reverse mutation.

These findings may have some bearing on the clinical use of ERY, where the aim is to kill bacteria without damaging mitochondria. Presumably rather similar mutational events would occur in bacteria and in mitochondria, though in the

former the phenotypic expression is only at the level of the individual bacterium, whereas in the latter it is at two levels – that of the individual mitochondrion and that of the cell, as previously discussed. It would be interesting to compare the effect of given concentrations of ERY, and other antibiotics having similar action, on both bacterial and mitochondrial mutations.

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