CHARACTERIZATION OF A MUTANT OF *CHLAMYDOMONAS REINHARDTII* RESISTANT TO FUSIDIC ACID

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1. Introduction

Fusidic acid, a steroid antibiotic, inhibits protein synthesis in vivo and in vitro by interacting with the elongation factors responsible for the translocation step (EF-2 of the cytoplasm of eukaryotes and EF-Gs of prokaryotes, mitochondria, and chloroplasts) [1]. However, experiments in vitro show the existence of conspicuous differences in the level of sensitivity to the antibiotic of the different types of translocases from different organisms or different cell compartments [2,3]. Thus, it appears that at a low concentration, fusic acid may be considered a specific inhibitor of organellar EF-Gs.

Here, mutants of *Chlamydomonas reinhardtii* resistant to fusidic acid were isolated with the aim of finding mutations in the genes for the organellar EF-Gs. Although resistance to fusidic acid was found to be inherited in a Mendelian fashion, only the organellar EF-Gs of the mutant strain appeared to be resistant in vitro to concentrations of the antibiotic inhibiting the EF-Gs from the parental strain. Thus the mutation appears to be localized in one of the genes for the organellar EF-Gs.

2. Materials and methods

2.1. *Organisms*

Wild-type mating-type plus (mt^+) and mating-type minus (mt^-) strains of *C. reinhardtii* were obtained from Dr L. Bogorad, Harvard University.

2.2. *Culture conditions*

Wild-type strains were grown in liquid cultures at 28°C in a high salt medium (HS) [4] supplemented with 0.1% (w/v) bacteriological peptone (Oxoid) under mixotrophic conditions (~4000 lux). The fusidic acid-resistant strain was cultured in the same medium supplemented with 800 μg fusidic acid/ml. Solid media were prepared by adding 1.5% (w/v) agar (IMA).

2.3. *Mutagenesis*

Wild-type mt^+ cells, grown to ~1×10^6 cells/ml, were collected by centrifugation, resuspended in HS medium (2.8×10^7 cells/ml) and treated with nitrosoguanidine (100 μg/ml for 45 min). Cells were then washed twice with HS medium and incubated in the light at 28°C for 24 h. After subculturing in HS medium supplemented with increasing concentrations of fusidic acid a mutant, TR-45, growing in the presence of 800 μg antibiotic/ml was isolated.

2.4. *Genetic analysis*

Cells of opposite mating-type, grown under mixotrophic conditions in solid medium, were resuspended in distilled water for gametogenesis. mt^+ and mt^- Gametes were mixed and allowed to mate for 4–6 h in the light. Zygotes were then transferred to solid medium and allowed to mature in the light at 28°C for 24 h and in the dark at 28°C for 6 days. Zygote germination was induced by exposure to light for ~24 h. For genetic analysis the 4 or 8 products of each zygote were separated by micromanipulation [5] and the level of resistance to fusidic acid assayed in solid medium.

2.5. *Preparation of soluble enzymes*

Cells were collected by centrifugation, washed and resuspended in 250 mM sucrose, 25 mM KCl, 25 mM Mg acetate, 25 mM Tris–HCl (pH 7.8), 6 mM 2-mercaptoethanol, 50 μM GTP (extraction buffer) and broken with an equal volume of sand in a cooled mor-

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tar. Following centrifugation at 27 000 × *g* for 30 min, the supernatant was recovered and glycerol was added to a final concentration of 10% (w/v). After ultracentrifugation at 100 000 × *g* for 2 h, the supernatant, containing crude soluble enzymes, was recovered and used immediately or stored at −70°C.

2.6. *Assay of cytoplasmic and organellar elongation factors*

Cytoplasmic and organellar elongation factors were assayed in crude extracts by measuring poly(U)-directed polyphenylalanine synthesis in standard mixtures containing [^{14}C]phenylalanyl-tRNA and yeast cytoplasmic or *Escherichia coli* ribosomes as in [6] except that the samples were incubated at 30°C for 30 min.

2.7. *Partial purification of EF-Gs*

The preparation of soluble enzymes was treated with solid ammonium sulphate (0–80% saturation at 0°C). After centrifugation at 13 000 × *g* for 15 min, the precipitate was dissolved in 0.15 M KCl, 0.02 M Tris–HCl (pH 7.8), 50 μM GTP, 1 mM DTT and dialyzed in the cold against the same buffer for ~2 h. Organellar EF-Gs were partially purified by chromatography on DEAE-Sephadex as in [7]. The fractions corresponding to the peak of EF-G activity, as detected by assaying polyphenylalanine synthesis in the presence of *E. coli* EF-T and ribosomes, were pooled and solid ammonium sulphate added to give a final saturation of 80% at 0°C. The protein precipitate was dissolved in ~1 ml extraction buffer.

3. Results and discussion

As reported in table 1, mutant TR-45 is resistant to concentrations of fusidic acid that are almost twice those inhibiting growth of the wild-type strain. The mutant strain appeared to be resistant even when grown in liquid medium or in minimal medium under strict autotrophic conditions. It may be added that when resistance was transfered by back-crossing to a mt$^-$ strain, no difference was observed in the degree of resistance.

Evidence for a Mendelian inheritance of resistance to fusidic acid and for independent segregation of the mating type and resistance genes was provided by the results of table 2.

To ascertain that resistance to fusidic acid was due to a mutation in the protein synthetic systems and not to other causes (e.g., change in cellular permeability, degradation of the antibiotic), the effect of the antibiotic of polyphenylalanine synthesis was assayed in crude or partially purified preparations from the wild-type and resistant strains. As depicted in fig.1, polyphenylalanine synthesis by yeast cytoplasmic ribosomes incubated with a crude preparation of cytoplasmic elongation factors (EF-1 and EF-2) was inhibited by fusidic acid to the same extent in the sensitive strain and in the resistant mutant. On the contrary, when the same extracts were tested on *E. coli* ribosomes to assay the activity of the organellar elongation factors, the preparations from the resistant mutant were found to be unaffected by concentrations of fusidic acid that clearly inhibited the analogous preparations obtained from the parental strain (fig.2). Indeed, at 10^{-5} M and 5×10^{-5} M fusidic acid, the activity of the elongation factors from the parental strain was inhibited ~50% and 75%, respectively, while no inhibition was observed in the case of the mutant.

To confirm that organellar EF-Gs are the site of

Table 1
Effect of fusidic acid on the relative growth of the wild-type and resistant strains of *Chlamydomonas reinhardtii*

Strain	Fusidic acid concentration (μg/ml)					
	0	200	400	600	800	1000
Wild-type mt$^+$	+++	++	+	±	–	–
Wild-type mt$^-$	+++	++	+	±	–	–
TR-45 mt$^+$	+++	++	++	++	++	+

Symbols represent: –, no growth; +, very slight growth; +, ++, +++, relative growth in increasing order

Table 2
Segregation of fusidic acid resistance

Cross	No. tetrads analyzed	Marker segregation	
		FUS^R:FUS^S	mt$^+$:mt$^-$
fus TR-45 mt$^+$ × w.t. mt$^-$	10	2:2	2:2
fus mt$^-$ × w.t. mt^{+a}	7	2:2	2:2

[a] A resistant offspring from the first cross has been backcrossed with a wild-type mt$^+$

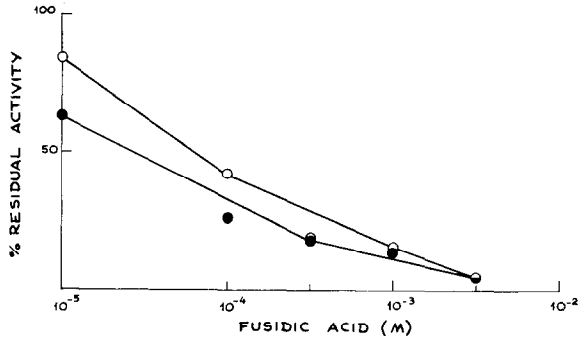

Fig.1. Effect of fusidic acid on polyphenylalanine synthesis by cytoplasmic elongation factors from the parental and mutant strains. The reaction mixtures contained (in 250 μl): 12.5 μmol maleate buffer (pH 6.6); 3 μmol magnesium acetate; 0.6 μmol β-mercaptoethanol; 0.25 μmol GTP; 1,25 μmol phosphoenolpyruvate; 8 μg pyruvate kinase; 10 μg spermine; 0.25 μmol reduced glutathione; 10 μg poly(U); 10 pmol *E. coli* L-[^{14}C]phenylalanyl-tRNA. *Chlamydomonas reinhardtii* soluble enzymes corresponding to 55 μg protein were added to each assay mixture containing 158 μg yeast cytoplasmic ribosomes and, when present, yeast enzymes corresponding to 203 μg protein. 100% corresponded to 3.5 and 2.4 pmol [^{14}C]phenylalanine incorporated in 30 min in the presence of enzymes from the wild-type (○) and resistant (●) strains, respectively.

action of fusidic acid, the same type of experiment depicted in fig.2 was repeated on partially purified preparations of EF-Gs. As this procedure does not allow one to separate chloroplasts EF-G from mito-

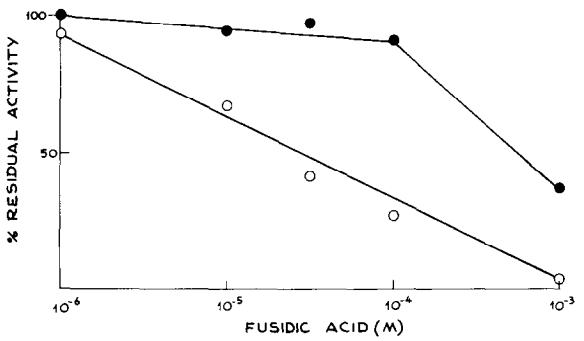

Fig.2. Effect of fusidic acid on polyphenylalanine synthesis by *E. coli* ribosomes in the presence of organellar EF-Gs from the parental and mutant strains. Reaction mixtures as in fig.1; *C. reinhardtii* soluble enzymes corresponding to 55 μg protein were added to each assay mixture in the presence of 137 μg *E. coli* ribosomes and *E. coli* EF-T (corresponding to 42 μg protein). 100% corresponded to 4.5 and 4.4 pmol [^{14}C]phenylalanine incorporated in 30 min in the presence of enzymes from the wild-type (○) and resistant (●) strains, respectively.

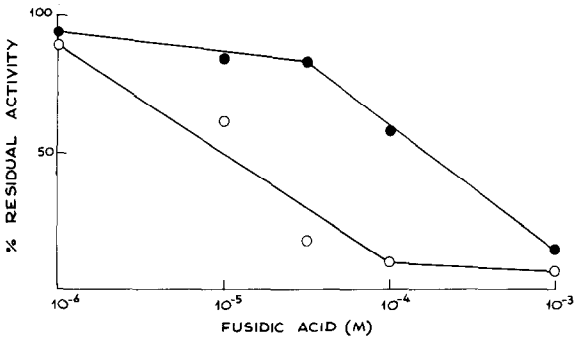

Fig.3. Effect of fusidic acid on polyphenylalanine synthesis by *E. coli* ribosomes in the presence of partially purified organellar EF-Gs. Reaction mixtures as in fig.1; *C. reinhardtii* EF-Gs corresponding to 104 μg and 90 μg protein from the wild-type or resistant strains, respectively, were added to each assay mixture containing 137 μg *E. coli* ribosomes and *E. coli* EF-T factor (corresponding to 42 μg protein). 100% corresponded to 6 and 4.7 pmol [^{14}C]phenylalanine incorporated in 30 min in the presence of EF-Gs from the wild-type (○) and resistant (●) strains, respectively.

chondrial EF-G [3], it is assumed that the partially purified preparations contain EF-Gs from both types of organelles. The data in fig.3 demonstrate that resistance or sensitivity to fusidic acid is maintained even in partially purified preparations of EF-Gs. Thus it appears quite likely that mutant TR-45 contains EF-Gs resistant to fusidic acid.

It is not yet possible to establish if the fusidic acid-resistant EF-G is that of the chloroplast or that of the mitochondrion or both. However, except in the case of *Euglena gracilis* [8], chloroplast EF-G has been shown to be synthesized and probably coded in the chloroplast in *Chlorella vulgaris* [7] and in spinach [9], while the mitochondrial one is a cytoplasmic product in *C. vulgaris* [10] and in fungi [11–13], coded in the nuclear DNA in yeast [11,12] and in *Neurospora crassa* [13]. Thus it is likely that the Mendelian mutation conferring resistance to fusidic acid in *C. reinhardtii* is probably affecting the gene for the mitochondrial elongation factor. If such were the case, it will be possible for the first time to map in an eukaryote the gene for a mitochondrial elongation factor.

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