A Method for Producing, Selecting, and Isolating Photosynthetic Mutants of *Euglena gracilis*

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ABSTRACT

A method was developed for the isolation of photosynthetic mutants of *Euglena gracilis*. It consists of the following steps. (*a*) Incubation of the cells under phototrophic conditions in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea for 1 week. This step caused a drastic reduction in the number of chloroplasts per cell; (*b*) mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine; (*c*) phototrophic growth for a few days to allow for phenotype expression; (*d*) selection by incubation in the presence of arsenate under phototrophic conditions for 2 days; (*e*) plating and growth under photoorganotrophic conditions; (*f*) assay of green colonies for ability to evolve oxygen. About 10% of the green colonies were found to be deficient in their ability to evolve oxygen. In principle the method may prove suitable for the isolation of other types of mutants of *Euglena*.

Euglena has been a favorite organism for photosynthetic studies mostly because of its ability to grow under phototrophic or organotrophic conditions, the complete dependence of the development of chloroplasts on light, and the ease of isolation of "clean" chloroplasts (15). However, in contrast to other popular algae, such as *Chlamydomonas* (9, 10) and *Scenedesmus* (13) mutants were only rarely employed in such studies. This is due mostly to the apparent lack of a sexual mode of reproduction with a haploidic phase during the life cycle of *Euglena* (2, 8), its higher nuclear ploidy (6), and the large number of chloroplast DNA complements (12). It is still not clear whether the genetic information which codes for the photosynthetic apparatus resides in the nuclear or in the chloroplast DNA or in both (15). In any case, in order to allow the induction of mutations, the nuclear or chloroplastic ploidy or both have been reduced.

This report describes a method (17) for isolating photosynthetic mutants of *Euglena* based on (*a*) a procedure for drastically reducing the number of chloroplasts per cell, (*b*) mutagenesis, (*c*) a procedure for selecting photosynthetic mutants.

MATERIALS AND METHODS

Euglena gracillis var. bacillaris was grown phototrophically with bubbling with 5% CO_2 (7) or photoorganotrophically in Hutner's acidic medium (5), with 160 ft-c, at 25 C on a rotary shaker. Plating was done following Lyman *et al.* (12).

DCMU was applied from a concentrated methanolic solution. Cell concentrations were determined by counting in a hemocytometer after addition of 2 drops of concentrated formic acid to 1 ml of cells to eliminate cell movement. The number of chloroplasts per cell was determined after fixation with polyvinyl alcohol as previously described (3). Oxygen evolution was determined with a Clark-type O_2 electrode using broad band red light of about 2×10^5 ergs cm^{-2} sec^{-1}.

RESULTS

Pretreatment of Cells for Mutagenesis. This step was designed to reduce nuclear and/or chloroplastic ploidy in order to obtain high yield of mutants. In a previous communication (18), we showed that the number of chloroplasts per cell sharply decreases on starving phototrophically grown cells in the presence of DCMU. This decrease in the number of chloroplasts per cell was accompanied by a parallel reduction in the number of cytoplasmic DNA targets involved in the formation of chloroplasts. Thus, starvation of phototrophically grown cells for 7 days in the presence of 20 μM DCMU at a cell concentration of 5×10^4 cells/ml comprised the first step in our procedure.

Mutagenesis. Mutagenesis with nitrosoguanidine[1] was performed essentially as described by Adelberg *et al.* (1). Pretreated cells were collected (1000g for 10 min), washed once with 50 mM sterile sodium citrate, pH 5, and suspended in the same buffer to give a final concentration of 10^8 cells/ml. Freshly prepared sterile nitrosoguanidine solution (0.22 ml of 0.5 mg/ml) was added to 2 ml of the cell suspension to give a final mutagen concentration of 50 μg/ml. Mutagenesis was allowed to proceed in the dark at 25 C with continuous shaking. Samples were taken out at different times, washed once with fresh phototrophic medium, and plated on organotrophic medium. After 8 days of incubation in the light, colonies were counted and the survival rate and percentage of green colonies were calculated.

The results of such an experiment conducted with cells grown phototrophically in the presence or absence of DCMU are shown in Figure 1. Whereas the sensitivity to the killing action of nitrosoguanidine was very similar in both types of cells, the ability to form green colonies was considerably more sensitive to the bleaching action of nitrosoguanidine in the DCMU-treated cells. These results may indicate a decrease in the number of cytoplasmic DNA targets involved in the formation of chloroplasts in the DCMU-treated cells, and thus would strengthen the previous conclusions based on the increased

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[1] Abbreviation: nitrosoguanidine: N-methyl-N'-nitro-N-nitrosoguanidine.

sensitivity of DCMU-treated cells to the bleaching effect of UV irradiation (19). The similar sensitivity of both types of cells to the killing effect of nitrosoguanidine might indicate an identical nuclear ploidy.

For all further work, we treated the cells with nitrosoguanidine for 25 min. After this period, the percentage survival of the DCMU-treated cells was about 20, 4% of which still formed green colonies. A sample of 2×10^6 DCMU-treated cells which were incubated with nitrosoguanidine for 25 min, were washed once with phototrophic medium (1000g for 10 min) then resuspended in 9 ml of the same medium contained in a 25-ml flask. The cells were incubated for 5 days in the light in equilibrium with 5% CO_2 in air. This incubation was made to allow for phenotypic expression of mutations before applying a selecting agent.

Selection. The basic idea behind a selection system for photosynthetic mutants was the inability of such cells to divide under phototrophic conditions. The efficiency of different compounds to discriminate between dividing and nondividing cells was monitored by applying them to cells grown phototrophically with or without DCMU. Of many potentially promising compounds, such as analogues of nucleic acids, amino acids, or vitamins, none turned out to be suitable for selection in *Euglena*. Surprisingly, arsenate which acts as an analogue of phosphate and so as an uncoupler of phosphorylation reactions (11) turned out to be the best selective agent (see also 20). Actidione, a protein synthesis inhibitor on 80S ribosomes (14), was second best, but much inferior. The results summarized in Table I show that 5 mM arsenate killed *Euglena* cells 20 times better in the absence of 20 μM DCMU than in its presence. Nevertheless, even in the presence of DCMU, the survival after 2 days was only about 20%. This relatively high killing of nondividing cells was found to be due to their preincubation for 2 days with DCMU prior to the addition of arsenate in order to starve them. DCMU by itself was found to markedly decrease the viability of *Euglena* cells under phototrophic growth conditions. This can be seen in the much better selective power which arsenate provides when a photosynthetic mutant (isolated by the procedure described herein) was used as a control (Table I). Cells of mutant 50 (18) which cannot grow under phototrophic conditions were virtually not affected by incubating them for 2 days in the presence of 25 mM arsenate. On the other hand, more than 99.5% of the wild type cells were killed under the same conditions. A concentration of 25 mM arsenate was found to be optimal for selection and was used thereafter.

Adopted Procedure. The final procedure adopted follows. (*a*) Cells were grown phototrophically. On reaching 5×10^4 cells/ml, 20 μM DCMU (in methanol) was added, and the cells were left under the same conditions for 7 days. They were washed once in 50 mM sodium citrate, pH 5 and resuspended in the same buffer to a final concentration of 10^6 cells/ml. (*b*) Freshly prepared sterile nitrosoguanidine (0.22 ml of 0.5 mg/ml) was added to 2 ml of cell suspension, and the suspension was continuously shaken in the dark at 25 C for 25 min. (*c*) The cells were washed once with phototrophic medium, resuspended in 9 ml of this medium and grown phototrophically for 5 days. (*d*) One ml of 250 mM sodium arsenate was added to the cell suspension. The cells were incubated for 2 days under phototrophic conditions. (*e*) The cells were washed once with phototrophic medium diluted with the same medium, plated on organotrophic medium (12), and incubated for 8 days in the light. (*f*) Green colonies were picked by eye, transferred into liquid heterotrophic medium and incubated in the light. The liquid cultures thus obtained were checked at the early logarithmic phase (5 μg Chl/ml) (see
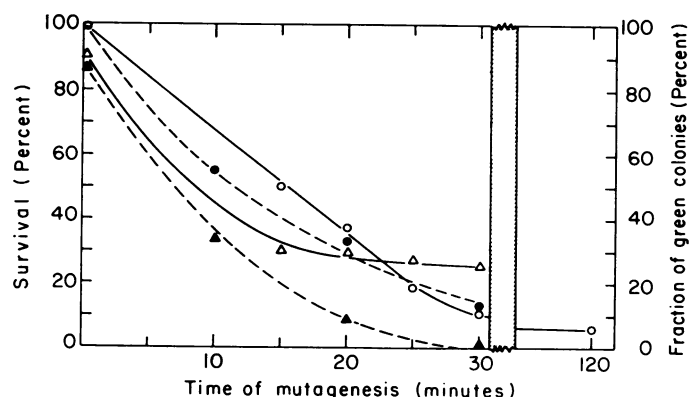

FIG. 1. Effect of nitrosoguanidine on the survival and the ability to form green colonies, of cells grown photoautotrophically in the presence or absence of DCMU. Survival of cells grown phototrophically in the absence (O) or presence (●) of DCMU. Fraction of green colonies formed by cells grown phototrophically in the absence (△) or presence (▲) of DCMU. Experimental details are given under "Materials and Methods."

Table I. *Selection of Photosynthetic Mutants by Arsenate and Actidione*

Wild type cells were grown phototrophically. Where indicated, 20 μM DCMU was added 48 hr prior to the selective agent. Mutant cells grown photoorganotrophically, were transferred to phototrophic media 3 days prior to the addition of the selective agent. Treatment with the selective agent was for 2 days.

Agent Added	Concn	Wild Type	Wild Type + DCMU	Mutant 50
			% survival	
Arsenate	5 mM	1.1	20	
Arsenate	25 mM	0.3		95
Actidione	40 μg/ml	0.6	3	

18). 5 to 15% of all the cultures showed a marked reduction in O_2 evolution in the light or no evolution at all.

DISCUSSION

A large number of green photosynthetic mutants of *Euglena* with different defects in their photosynthetic apparatus were isolated by the procedure described herein (17). The high yield of mutants can be related to two factors: (*a*) The marked decrease in chloroplast ploidy prior to mutagenesis; (*b*) the selection by arsenate. As no reduction in nuclear ploidy was observed in the DCMU-treated cells (as judged by the constant amount of total DNA per cell) the possibility that a reduction in chloroplast ploidy increased the yield of photosynthetic mutants should be considered. As described previously (18), DCMU-treated cells contained a reduced average number of chloroplasts. What seems even more important is the observation that a substantial number of these cells contained only one chloroplast. Obviously these cells were the best candidates for a successful mutagenesis. Russell and Lyman (14) have isolated one type of photosynthetic mutant of *Euglena* without any pretreatment or selection, by the use of UV or nitrosoguanidine. They proposed that UV irradiation or treatment of the cells with nitrosoguanidine inhibits division of chloroplasts. A few cells remain with a single chloroplast which retains its capacity to divide, and these after mutation formed a homogeneous population of mutants.

The ability of arsenate to discriminate between dividing and nondividing cells was unexpected. Nevertheless, it proved to be a good selective agent killing dividing cells 300 times more efficiently than nondividing cells. Moreover, the survival rate of the nondividing cells was very high. Arsenate was found to be an efficient selective agent also in the case of *Chlamydomonas reinhardi*, enabling the isolation of a number of auxotrophic mutants requiring amino acids other than arginine (A. Shneyour *et al.*, unpublished results). In this case, it could be directly demonstrated that it affected essentially only dividing cells. Addition of the required amino acid to an auxotrophic mutant, which restored cell division, brought about a high rate of mortality in the presence of arsenate.

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