Nitrate Reductase Deficient Mutants of *Chlamydomonas reinhardtii*. Isolation and Genetics

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Following u.v.-irradiation and plating on a selective chlorate medium, about 200 chlorate-resistant isolates of *Chlamydomonas reinhardtii* were obtained. These were divided into three groups all of which grew well with ammonium as nitrogen source. One group grew well with nitrate under all conditions tested. The second, and largest group, grew well with nitrate only in the absence of acetate. The third group could not grow with nitrate as sole nitrogen source. Isolates in this group were further subdivided according to their ability to grow with hypoxanthine or nitrite as sole nitrogen source. Three mutants (14/15, 17/4 and 0/8) were examined genetically in some detail. Each differed from wild-type (strain 2192) by a mutation in a single gene. Isolates 14/15 and 17/4 could grow with hypoxanthine as a nitrogen source. The mutant genes in these two isolates were shown to be located in different linkage groups and have been designated *nitA* (in 17/4) and *nitB* (in 14/15). There is evidence that *nitA* may be in linkage group VI. Mutant 0/8 resembles the *cnx* mutants of *Aspergillus* in that it would not grow on hypoxanthine. The mutation in 0/8 is designated *nitC*; it, too, may be in linkage group VI. Strain 137c was also analysed. It appears to be unable to grow on nitrate because of mutations in two loci.

INTRODUCTION

The biochemistry and physiology of nitrate reduction by algae has received much attention, particularly in recent years (see, for example, Hewitt, 1975; Syrett & Leftley, 1976). Our knowledge of this process in organisms other than algae, more especially in bacteria and fungi, also owes much to the methods of biochemical genetics (Stouthamer, 1976; Cove, 1970). Limited use of these techniques has been made in studies of nitrate assimilation by blue-green algae (Delaney *et al.*, 1976) but nothing has been done with eukaryotic algae. The application of these methods to photosynthetic organisms is desirable because of the close interrelationships, in algae, between nitrate reduction and photosynthesis (Syrett & Leftley, 1976). The alga that has been most used in genetical studies is *Chlamydomonas reinhardtii* and much is known about its genetics (Hudock & Rosen, 1976). Thacker & Syrett (1972*a, b*) studied the biochemistry and physiology of nitrate assimilation by *C. reinhardtii* and nitrate and nitrite reductases from it have been purified and characterized (Earea & Cárdenas, 1975). It is clear that in *C. reinhardtii*, as in other organisms, nitrate reduction takes place in two stages: firstly, the reduction of nitrate to nitrite catalysed by an NADH-dependent nitrate reductase and, secondly, the reduction of nitrite to ammonium catalysed by a ferredoxin-dependent nitrite reductase. In this paper we describe the isolation and genetics of mutants of *C. reinhardtii* that are unable to grow with nitrate as nitrogen source. The mutants were isolated as organisms able to grow on a chlorate medium. This technique

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has been used successfully to obtain mutants defective in nitrate assimilation from other organisms (Stouthamer, 1967; Cove, 1976*a*). In the following paper (Nichols *et al.*, 1978) we describe some biochemical properties of these mutants.

METHODS

Organisms. *Chlamydomonas reinhardtii* strain 2192, mating type plus (+), and its complementary minus (−) mating type, 6145, were obtained from Professor Ruth Sager. Strains 2192 and 6145 are thought to be genetically identical except for the mating type (*mt*) alleles and their dark growth characteristic (*y*-*I*). Strain 2192 is green when grown in the light or dark (*y*⁺-*I*), whilst 6145 is yellow under dark growth conditions (*y*[−]-*I*) because of little or no chlorophyll formation (Sager, personal communication; Sager, 1955). Both mating types grow well with either nitrate-nitrogen or ammonium-nitrogen as sole nitrogen source.

Strain 137c [both plus (+) and minus (−) mating types] was obtained from Professor R. P. Levine. This strain is able to utilize ammonium-nitrogen for growth, but not nitrate-nitrogen. There is very close nuclear homology between 137c and the 'Sager' strain, both mating freely with each other (Sager, 1972).

Medium and growth conditions. The liquid minimal medium was based on the high salt minimal (HSM) medium of Sueoka (1960) with either NH_4Cl (0.5 g l^{-1}) or KNO_3 (0.94 g l^{-1}) as a nitrogen source. For solid media, 1.5% (w/v) Difco agar was added. Modifications to this basic medium are indicated, as appropriate, in the text. All cultures were grown at 25 °C under continuous illumination from a mixture of warm white and daylight fluorescent tubes. Unless indicated otherwise, the light intensity was 30 W m^{-2} .

Induction and isolation of mutants. Strain 2192 was grown in HSM medium with nitrate as nitrogen source, with aeration [30 l h^{-1} ; air supplemented with 0.5% (v/v) CO_2], until the cells were in mid-exponential phase (about 72 h). Organisms were harvested by centrifugation, washed with, and resuspended in, nitrogen-free minimal medium (HSM-N). Samples (12 ml) were irradiated with u.v. light from a Hanovia 11a mercury discharge tube at a dose rate of $2.2 \text{ J m}^{-2} \text{ s}^{-1}$, to give approximately 10% cell survival (about 95 s under the conditions employed). All post-irradiation operations were carried out in red light to prevent photoreactivation.

Irradiated samples, after dilution with HSM-N medium, were plated to provide a lawn of cells on chlorate minimal medium and on chlorate complete medium (see below). After 24 h in darkness, the plates were illuminated at 11.2 W m^{-2} for up to 28 d before being examined for the presence of green, actively growing colonies amongst a background of dead cells. These colonies were taken to be presumptive mutants. After re-isolation from pure single colonies, all isolates were coded and maintained on slopes of ammonium-HSM medium supplemented with sodium acetate (2 g l^{-1}). The composition of the selective chlorate media was essentially the same as that of the nitrate-HSM medium, except (i) the KNO_3 concentration was increased fivefold (to 4.7 g l^{-1}) and (ii) KClO_3 (10 g l^{-1}) and sodium acetate (2 g l^{-1}) were added. An alternative nitrogen source to nitrate was also included; this was urea (0.03 g l^{-1}) in chlorate minimal medium and yeast extract (4 g l^{-1}) in chlorate complete medium.

Growth tests with presumptive mutants. Each mutant was tested initially for growth in liquid HSM medium with either nitrate or ammonium as sole nitrogen source. Both media were used with and without acetate. Mutants unable to grow on nitrate were tested for their ability to utilize sodium nitrite (0.65 g l^{-1}), hypoxanthine (0.32 g l^{-1}) or uric acid (0.40 g l^{-1}) as sole nitrogen source, again in the presence or absence of acetate. The inoculum for growth tests was always prepared by culturing a mutant for 5 d at 11.2 W m^{-2} light intensity on solid ammonium-HSM medium followed by transfer of organisms to liquid HSM-N medium for 4 h. This procedure produced an inoculum of unclumped cells in a medium reasonably free of nitrogen. Portions (0.25 ml ; about 2×10^8 cells) were inoculated into 30 ml of the appropriate medium in 100 ml Erlenmeyer flasks, and the cultures grown at a light intensity of 12.5 W m^{-2} . Growth was scored visually over 14 d. In all growth tests strains 137c(+) and (−) were included, together with strains 2192 and 6145, as controls.

Genetic analysis. Gametogenesis, mating, zygote maturation and tetrad analysis were carried out using, with modification, the basic techniques developed by several workers (Sager & Granick, 1954; Sager, 1955; Levine & Ebersold, 1958, 1960). To induce gamete formation, cultures were grown on gametogenesis medium A (GM-A) or gametogenesis medium B (GM-B). Both media were the same as the low phosphate minimal medium of Sueoka (1960) except that (i) NH_4NO_3 was used as the nitrogen source at 60 mg l^{-1} for GM-A and at 20 mg l^{-1} for GM-B and (ii) sodium acetate (2 g l^{-1}) was added. Strains able to utilize only ammonium-nitrogen were grown on GM-A, whilst strains able to utilize nitrate or ammonium were grown on GM-B. Vegetative cultures were grown for 5 d before being transferred to darkness overnight. Plates were then flooded with nitrogen-free GM-A medium. After 4 to 5 h illumination (21.5 W m^{-2}), equal volumes of cell suspensions of opposite mating types were mixed and left for a further hour before 0.2 ml samples were plated on to minimal maturation medium (HSM medium with NH_4Cl as nitrogen source). Plates were

Table 1. *Classification of chlorate resistant isolates of Chlamydomonas reinhardtii after initial growth tests with nitrate or ammonium as sole nitrogen source*

Liquid HSM medium was used with either nitrate (NO_3) or ammonium (NH_4) as sole nitrogen source, and with acetate (+Ac) or without acetate (–Ac). Cultures were grown at 25 °C under a light intensity of 12.5 W m^{-2} . Growth was estimated visually after 14 d. 0, no growth; $\pm$, negligible, but detectable growth; +, growth comparable with that of the wild-type strains (2192 and 6145) on the same medium. The number of isolates in each group is shown in the right-hand column. Growth tests with strains 2192, 6145 and 137c were included as controls.

Group	Additions to liquid HSM medium				No. of isolates
	NO_3 + Ac	NO_3 – Ac	NH_4 + Ac	NH_4 – Ac	
I	+	+	+	+	29
IA	$\pm$	+	+	+	12
IB	$\pm$	$\pm$	+	+	13
II	0	+	+	+	72
IIA	0	$\pm$	+	+	47
III	0	0	+	+	39
Strain 137c(+) or 137c(–)	0	0	+	+	
Strain 2192 or 6145	+	+	+	+	

incubated at a light intensity of 30 W m^{-2} for 18 to 20 h before being transferred to darkness for 6 d. Mature zygotes (with unmated vegetative cells) were then removed and streaked on to fresh germination medium [maturation medium containing 2.2% (w/v) agar] and, after zygote separation and a 40 s exposure to chloroform vapour to kill vegetative cells, the plates were illuminated for 18 to 24 h to initiate synchronous zygote germination. The four or eight meiotic products produced by the germinating zygotes were separated from the zygotes and from each other, without removal from the plates. Further incubation of the plates in light for 5 to 7 d produced small colonies visible to the naked eye.

The individual colonies from each zygote were picked off and cultured on ammonium-HSM medium, before being replica-plated on to nitrate-HSM medium. Mating type was determined by the method of Sager (1955). The 'yellow in the dark' characteristic was determined by growing the meiotic products in light or in darkness on ammonium-HSM medium containing sodium acetate (2 g l^{-1}).

RESULTS

Isolation and classification of mutants

From the chlorate plates about 200 colonies were isolated and it was expected that some of these would be mutants unable to utilize nitrate. The isolates were tested for their ability to grow in liquid nitrate or ammonium media with or without sodium acetate. The results are summarized in Table 1. All isolates could grow with ammonium as nitrogen source. Some (group I) grew well with nitrate as nitrogen source irrespective of whether acetate was added; others (group IA) grew well with nitrate only in the absence of acetate. Group IB mutants grew poorly with nitrate but made sufficient growth to distinguish them from mutants in groups II and III. A large group of isolates (group II) grew well on nitrate in the absence of acetate but made no growth in its presence; a smaller group (group IIA) made poorer growth on nitrate but only in the absence of acetate. The final group of isolates (group III) could not grow with nitrate as nitrogen source either with or without acetate.

Further work was carried out only with isolates of group III. These were tested for their abilities to grow with nitrite, hypoxanthine or uric acid as sole nitrogen sources. The results are shown in Table 2. Growth tests were made both in the presence and absence of acetate. In general, addition of acetate had little effect, but it slightly reduced growth of some of the isolates on hypoxanthine. These tests allowed the separation of the group III mutants into four sub-groups. The first (sub-group IIIA) contained isolates which could grow with

Table 2. *Classification of Chlamydomonas reinhardtii* mutants unable to grow on nitrate (group III mutants) according to ability to grow with nitrite, hypoxanthine or uric acid as nitrogen source

Liquid HSM medium was used with nitrate (NO_3), nitrite (NO_2), hypoxanthine (Hyp) or uric acid (UA) as sole nitrogen source, and with or without acetate ($\pm$ Ac). See Table 1 for further details.

Sub-group	Additions to liquid HSM medium				No. of isolates
	$\text{NO}_3 \pm \text{Ac}$	$\text{NO}_2 \pm \text{Ac}$	Hyp $\pm$ Ac	UA $\pm$ Ac	
IIIA	0	+	+	+	7
IIIB	0	+	0	+	15
IIIBi	0	+	$\pm$	+	1
IIIC	0	0	+	+	6
Strain 2192 or 6145	+	+	+	+	
Strain 137c(+) or 137c(-)	0	+	+	+	

either nitrite, hypoxanthine or uric acid as sole nitrogen source. The second (sub-group IIIB) grew on nitrite and uric acid but not on hypoxanthine; one isolate (placed in sub-group IIIBi) was similar to these but made a little poor growth on hypoxanthine. The fourth sub-group (IIIC) grew on hypoxanthine and uric acid but not on nitrite. Three mutants gave growth patterns which could not be classified.

Genetic analysis

Genetic analysis was carried out with two isolates (designated 14/15 and 17/4) from subgroup IIIA and one isolate (0/8) from subgroup IIIB. Strain 137c was also analysed; its growth behaviour was similar to that of isolates in subgroup IIIA (Table 2). Failure to grow on nitrate is termed *nit*⁻.

The techniques used for genetical analyses were checked by crossing the Sager wild-type strains, i.e. 2192 (*mt*⁺, *y*⁺-*I*) with 6145 (*mt*⁻, *y*⁻-*I*), and analysing, by tetrad analysis, the meiotic products for mating type and the 'chlorophyll in the dark' phenotype. Sixteen tetrads were analysed and in all these both mating type and the 'chlorophyll in the dark' character segregated with 2:2 ratios. Of the 16 tetrads, 10 (62.5%) were tetratype (T), 5 (31%) parental ditype (PD) and 1 (7%) non-parental ditype (NPD). Although the numbers analysed were small, the percentage of tetratypes agreed well with the 63.6% found by Sager (1955) in a similar cross. This result confirms that the markers *mt* and *y*-*I* are unlinked as reported previously (Sager, 1955).

Cross 1: 17/4 (*mt*⁺, *nit*⁻) $\times$ 6145 (*mt*⁻, *nit*⁺); 32 tetrads resulting from this cross were analysed. All showed 2:2 segregation of mating type and 2:2 segregation of ability to grow on nitrate. The *nit*⁻ characteristic in 17/4, therefore, clearly results from a mutation in a single Mendelian gene. The numbers of different types of tetrads were 5PD:5NPD:22T. A PD:NPD ratio of unity is generally indicative of non-linkage between the two genes but it may also result from loose linkage; if this is so, a T:NPD ratio of greater than 4 is expected and the frequency of tetratypes should be approximately 67% (Perkins, 1953; Barratt *et al.*, 1954). In this cross the T:NPD ratio is 22:5, i.e. 4.4, and the tetratype frequency is 68.7%; it may be, therefore, that the *nit*⁻ mutation in 17/4 is loosely linked to the *mt* locus. Hereafter the gene carrying the *nit*⁻ mutation in 17/4 is designated as *nitA*.

Cross 2: 14/15 (*mt*⁺, *nit*⁻) $\times$ 6145 (*mt*⁻, *nit*⁺); 31 tetrads were analysed. Again both the mating type character and ability to grow on nitrate segregated with 2:2 ratios. Isolate 14/15, therefore, also carries a single gene mutation for nitrate metabolism. The numbers of different types of tetrads were 6PD:6NPD:19T. A PD:NPD ratio of unity and a T:NPD

Table 3. Analysis of 21 tetrads from the cross 0/8 (mt^+ , $nitC^-$, y^+-I) $\times$ 6145 (mt^- , $nitC^+$, y^-I)

Mendelian gene pairs	Tetrad type			% T	T:NPD	Percentage recombination*
	PD	NPD	T			
$nitC:y-I$	6	4	11	52.3	2.75	50.0
$nitC:mt$	5	2	13	65.0	6.50	42.5
$y-I:mt$	4	6	11	52.3	1.83	54.7

* Calculated as $[(\frac{1}{2}T + NPD)/(T + NPD + PD)] \times 100$.

ratio of less than 4 indicate that in 14/15 the mt locus and the nit^- mutation are not linked. This result suggests that this mutation is in a gene other than $nitA$; it is here designated as $nitB$.

Cross 3: 14/15 (mt^+ , $nitB^-$, $nitA^+$) $\times$ 17/4 (mt^- , $nitB^+$, $nitA^-$). Confirmation that the mutations in 14/15 and 17/4 are different was obtained from this cross. The 47 tetrads analysed were made up of 10PD, 12NPD and 25T with respect to ability to grow on nitrate; the mating type character segregated 2:2 in all tetrads. The nature of the tetrads was determined from the distribution among the meiotic products of ability to grow on nitrate. Neither of the parents could grow with nitrate as nitrogen source. Therefore tetrads from which no meiotic products could grow on nitrate were PD tetrads. Tetrads in which two out of four products could grow on nitrate must have arisen from recombination and were NPD tetrads. Similarly those in which one out of four products could grow on nitrate must have been tetratype (T). The ratio of PD:NPD tetrads did not differ significantly from unity and the T:NPD ratio was 2.1. These are the results expected from the independent assortment of two genes both of which are essential for growth on nitrate.

Cross 4: 137c (mt^+ , nit^- , y^+-I) $\times$ 6145 (mt^- , nit^+ , y^-I); 33 tetrads were analysed. Again both the mating type and 'chlorophyll in the dark' characters each segregated with 2:2 ratios. For the nit^- phenotype, 6 tetrads segregated 4 nit^- :0 nit^+ , 21 segregated 3 nit^- :1 nit^+ and 6 segregated 2 nit^- :2 nit^+ . Since there is no evidence of abnormal segregation of the markers mt and $y-I$, the simplest interpretation of these results is that there are two mutant loci associated with nitrate assimilation in our strain of 137c.

Cross 5: 17/4 (mt^+ , $nitA^-$) $\times$ 137c (mt^- , nit^-). **Cross 6:** 14/5 (mt^+ , $nitB^-$) $\times$ 137c (mt^- , nit^-). In both of these crosses, although more than 90% of zygotes germinated, only about 10% of them produced viable meiotic products. Determination of the mating type of viable progeny from both complete and incomplete tetrads showed that loss of viability was occurring randomly. From neither cross could any wild-type recombinants be recovered either by tetrad analysis or by a limited number of experiments involving random zygote analysis. The results from these two crosses, taken together, suggest that not only does strain 137c possess two mutant loci but that one of these is allelic or very closely linked to $nitA$ and the other is similarly related to $nitB$.

Cross 7: 0/8 (mt^+ , nit^- , y^+-I) $\times$ 6145 (mt^- , nit^+ , y^-I); 21 tetrads were analysed. Mating type and 'chlorophyll in the dark' each segregated 2:2 as normal, as did ability to grow on nitrate. Consequently isolate 0/8 possesses a single mutation for nitrate assimilation. This isolate was, however, unlike either 14/15 or 17/4, because it could not grow on hypoxanthine. It is reasonable to assume, therefore, that the mutant locus in 0/8 differs from $nitA$ and $nitB$. It is designated tentatively as $nitC$. The distributions of PD, NPD and T tetrads derived from this cross (Table 3) rule out linkage between the $nitC$ and $y-I$ loci. There may, however, be loose linkage between $nitC$ and the mt locus because, although the number of tetrads analysed was small, there was an excess of PD tetrads over NPD ones, the T:NPD ratio was 6.5, and the percentage recombination was 42.5. The results also confirm non-linkage between the $y-I$ and mt loci.

DISCUSSION

Three major groups of mutants of *C. reinhardtii* were obtained by plating a u.v.-irradiated suspension of organisms on a chlorate medium. One group of mutants (group III) was of the type we were seeking, namely those unable to grow with nitrate as nitrogen source. The usual explanation for the isolation of this sort of mutant on a chlorate medium is that organisms with an effective nitrate reductase enzyme reduce chlorate to chlorite because chlorate is an alternative substrate for the enzyme (Solomonson & Vennesland, 1972). Chlorite is toxic and hence these cells are killed. Mutants lacking nitrate reductase activity survive and will grow if the medium contains a source of nitrogen such as urea or yeast extract. This explanation has been questioned by Cove (1976*b*) who points out that not all mutants of *Aspergillus* that lack nitrate reductase are chlorate resistant. Moreover, it is clear that there are other mechanisms for chlorate resistance. We isolated a group of mutants (group I) which were resistant to chlorate but which grew well on nitrate. Our largest group of mutants, however, consisted of organisms which would grow on nitrate in the absence of acetate but not in its presence. We have no explanation for this somewhat unexpected growth behaviour and have not pursued it. It clearly merits further study.

Our mutants of group III were further subdivided according to their ability to grow with nitrite or hypoxanthine as nitrogen source. Some (sub-group IIIA) could grow with either compound, some (sub-group IIIB) only with nitrite and some (sub-group IIIC) only with hypoxanthine. Failure to grow with either nitrate or hypoxanthine as a nitrogen source is characteristic of the *cnx* mutants of *Aspergillus* (Pateman *et al.*, 1964) and of the *nit-1* mutants of *Neurospora* (Coddington, 1976). These mutants lack a molybdenum-containing cofactor which is a constituent both of nitrate reductase and xanthine dehydrogenase (Arst *et al.*, 1970). We analysed genetically only one mutant (0/8) from this group. It has a mutation in a single Mendelian gene (tentatively designated *nitC*); this may be loosely linked to the mating type locus (*mt*) which is known to be located in linkage group VI in *C. reinhardtii* (Ebersold *et al.*, 1962).

Genetic analysis was also carried out on two mutants 14/15 and 17/4 from sub-group IIIA, i.e. organisms able to grow on nitrite or hypoxanthine but unable to grow on nitrate. Each of these organisms differed from wild type by a single mutation but these mutations were genetically distinct and the alleles containing them segregate independently. The mutant gene in 17/4, designated *nitA*, may be linked loosely with the mating type locus (*mt*). If so, it, like *nitC*, is in linkage group VI. The mutant gene in 14/15, designated *nitB*, is linked neither with the mating type locus nor with the gene (*y-1*) controlling chlorophyll formation in darkness. Further information on the biochemical properties of these mutants is given in the following paper (Nichols *et al.*, 1978).

We also examined genetically *C. reinhardtii* strain 137c. This strain which has been extensively used by Levine and his colleagues in studies of the biochemical genetics of the photosynthetic mechanism (Levine & Goodenough, 1970) is unable to grow on nitrate. Our results indicate that it probably differs from the Sager strains (2192 and 6145) by mutations in two loci for nitrate assimilation. One of these appears to be identical with or closely linked to *nitA* and the other may be identical or closely linked to *nitB*. A double mutation in strain 137c may account for the failure of Rosen (quoted in Hudock & Rosen, 1976) to isolate nitrate utilizing strains by reverse mutation.

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