Mitochondrial Genetics of *Chlamydomonas reinhardtii*: Resistance Mutations Marking the Cytochrome *b* Gene

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ABSTRACT

We describe the genetic and molecular analysis of the first non-Mendelian mutants of *Chlamydomonas reinhardtii* resistant to myxothiazol, an inhibitor of the respiratory cytochrome *bc1* complex. Using a set of seven oligonucleotide probes, restriction fragments containing the mitochondrial cytochrome *b* (*cyt b*) gene from *C. reinhardtii* were isolated from a mitochondrial DNA library. This gene is located adjacent to the gene for subunit 4 of the mitochondrial NADH-dehydrogenase (*ND4*), near one end of the 15.8-kb linear mitochondrial genome of *C. reinhardtii*. The algal cytochrome *b* apoprotein contains 381 amino-acid residues and exhibits a sequence similarity of about 59% with other plant cytochrome *b* proteins. The *cyt b* gene from four myxothiazol resistant mutants of *C. reinhardtii* was amplified for DNA sequence analysis. In comparison to the wild-type strain, all mutants contain an identical point mutation in the *cyt b* gene, leading to a change of a phenylalanine codon to a leucine codon at amino acid position 129 of the cytochrome *b* protein. Segregation analysis in tetrads from reciprocal crosses of mutants with wild type shows a strict uniparental inheritance of this mutation from the mating type minus parent (UP^-). However, mitochondrial markers from both parents are recovered in vegetative diploids in variable proportions from one experiment to the next for a given cross. On the average, a strong bias is seen for markers inherited from the mating type minus parent.

IN contrast to the very large mitochondrial DNA molecules of higher plants, the mitochondrial genome of *Chlamydomonas reinhardtii* is a small linear DNA molecule of 15.8 kb present in about 50 copies per cell (for a review, see HARRIS 1989). To date, about 90% of this mitochondrial DNA sequence has been determined (PRATJE, SCHNIERER and DUJON 1984; VAHRENHOLZ *et al.* 1985; BOER and GRAY 1986, 1988a,b; KÜCK and NEUHAUS 1986; PRATJE *et al.* 1989), thus allowing the identification of several genes by their similarity with homologous mammalian or plant mitochondrial genes. Only the DNA sequences of those genes which are located on the very terminal regions of the linear genome have not yet been determined. This is due to difficulties in cloning terminal fragments from linear DNA genomes. Although numerous attempts have been made to develop mitochondrial genetics in *C. reinhardtii*, our knowledge of this field remains sketchy: a complex non-Mendelian pattern of transmission was observed in tetrad analysis for "minute" colonies displaying a specific loss of mitochondrial DNA (ALEXANDER, GILLHAM and BOYNTON 1974; GILLHAM, BOYNTON and HARRIS 1987). Uniparental transmission of the mitochondrial genome was observed in tetrads from the mating type minus parent (UP^-) in crosses between the two interfertile species *C. reinhardtii* and *Chlamydomonas smithii* (BOYNTON *et al.* 1987). Vegetative diploids selected from reciprocal crosses between these two species showed unidirectional transfer of a unique 1-kb insertion of the mitochondrial genome belonging to *C. smithii* and biparental transmission of other physical markers. A dark-dier mutant which carries a 1.5-kb deletion in the vicinity of the *cyt b* gene displayed a UP^- mode of inheritance in random spore analysis of crosses to wild type (MATAGNE *et al.* 1989)

A more precise knowledge of mitochondrial genetics in *C. reinhardtii* requires mutations marking different mitochondrial genes to carry out extensive analysis of segregation and recombination in tetrads and vegetative diploids. The first point mutation in the gene encoding cytochrome *b* was selected and analyzed here by means of genetic and molecular techniques. With the aid of a set of different oligonucleotides and using polymerase chain reaction (PCR) methodology, we were successful in isolating the wild-type gene encoding cytochrome *b*. The primary sequence of this *cyt b* gene provided the basis for a molecular and genetic analysis of mutations conferring resistance to myxothiazol, a quinol oxidation inhibitor of cytochrome *b* (VON JAGOW and LINK 1986).

MATERIALS AND METHODS

Strains and culture conditions: Several strains of *C. reinhardtii* were used in this work: wild-type isolates mt^+ and

mt^- derived from 137C; the closely linked arginine auxotrophic strains *arg-2* and *arg-7*, mt^+ and mt^- (EVERSOLE 1956; LOPPES 1969); the closely linked nonphotosynthetic strains *F22* and *F31*, mt^+ and mt^- (GIRARD *et al.* 1980); the *cw15* mt^- (CC-406) cell wall less strain supplied by the *Chlamydomonas* Genetics Center (Department of Botany, Duke University, Durham, North Carolina). *Escherichia coli* strain J 83 was used for cloning with the bacterial vector pUC8 (VIEIRA and MESSING 1982); strain SMH50 (Eckstein, personal communication) was used for propagation of derivatives from single stranded phage M13mp19 (NORRANDER, KEMPE and MESSING 1983). *Chlamydomonas* cells were grown at 25° on Tris-acetate-phosphate (TAP) medium (GORMAN and LEVINE 1965) or minimal (HS) medium (SUEOKA 1960); gametes were differentiated under an illumination of 150 lux on acetate (HSA) medium (SUEOKA 1960) in which the amount of nitrogen was reduced tenfold. *E. coli* cells were grown at 37° in Luria-Bertani (LB) medium with 50 μg/ml ampicillin (SAMBROOK, FRITSCH and MANIATIS 1989).

Selection of vegetative diploids: Vegetative diploids were selected using either the complementing nuclear nonphotosynthetic mutants *F22* and *F31* or the complementing auxotrophs *arg-2* and *arg-7* (EBERSOLD 1963). A control for diploidy was achieved by estimating the chlorophyll content per cell for each clone selected. Diploid cells are larger than haploid cells and therefore possess a higher chlorophyll content. Cells were counted with a hemocytometer following growth in liquid TAP medium at 4000 lux until the end of exponential phase (around 1.2×10^7 haploid cells/ml or 8×10^6 diploid cells/ml). An aliquot of the cell suspension was diluted with dimethyl-sulfoxide 70% vol/vol and the optical density of the mixture recorded at 680 nm. Cell lines were considered as diploid when they displayed a ratio of this OD over the cell density equal to or higher than 6×10^{-8}. The ratio currently observed for haploid cells was between 2 and 4×10^{-8}, whereas it was between 5 and 10×10^{-8} for diploid cells. A control for mating type was also performed to ensure that the presumptive diploids were functionally mt^-, since mt^- is dominant to mt^+ with regard to mating (HARRIS 1989)

Haploidization of diploid strains: Diploid were crossed with a wild-type haploid mt^+ strain and haploid or aneuploid isolates were recovered in the progeny either as arginine requiring colonies or as high fluorescence colonies (BENNOUN and CHUA 1976) depending on the markers used for diploid selection. These isolates were back-crossed to wild type before use to ensure they were behaving as proper haploids.

Selection of mucidin-resistant strains: Isolates DPMU1, DPMU2, DPMU3 and DPMU4 were selected as diploid colonies growing in the dark on TAP medium supplemented with 8×10^{-7} M mucidin. They appeared as spontaneous mutants in 10^8 cells screened from cultures of independent diploid *arg-2 arg-7* strains and grow heterotrophically on 10^{-6} M mucidin and 10^{-6} M myxothiazol. Wild-type diploid and haploid cells are killed by 10^{-7} M mucidin and 4×10^{-8} M myxothiazol under heterotrophic conditions.

DNA isolation from *C. reinhardtii* strains: Large scale DNA isolation was performed as already described by KÜCK and NEUHAUS (1986). Small scale DNA isolation from mutant strains was done according to the procedure described by ROCHAIX *et al.* (1989).

Gel electrophoresis, hybridization conditions, DNA sequencing and standard *in vitro* recombinant DNA: These procedures were carried out as previously described (KÜCK *et al.* 1987; KÜCK 1989).

Oligonucleotides used for PCR and sequencing: Nucleotide positions shown below are from the *cyt b* DNA sequence which has been reported to the EMBL data library (accession number X51481), except for position of oligonucleotide 184 which is from PRATJE *et al.* (1989).

No.	Sequence	Position
151	5′-GTT (GA)GT (AT)AC (AG)GT (AG)GC (AG)CC CCA	469–449
152	5′-TTT GT(CT) TGG (GA)AT (GA)CT (AG) CG CA(GA) (AG)AT	889–866
158	5′-TTT ATT GTA GTC TAT TTG CAC	293–313
167	5′-CAA GGA AAG TCC AGT ATA GCC AC	1007–991
169	5′-GCC ATT CCA GTA GTA GGT AA	482–501
184	5′-TTG CTA ATT GGT GCT GTA TGG CTA	1288–1311
191	5′-GAA CCG CGT ACC GTA AGT GTA AA	1214–1192

Polymerase chain reaction: *Cyt b* DNA was amplified using a Bioexcellence cycler (Biozym, Hameln). About 5 μg of total DNA from various *C. reinhardtii* strains was used for amplification according to the manufacturer's protocol. The conditions used for PCR were: denature 92°, 2 min, primer anneal 60°, 2 min, primer extension 72°, 4 min, 30 cycles.

Chemicals: Myxothiazol was purchased from Boehringer Mannheim. Mucidin was a gift from G. DUJARDIN (C.G.M., 91190 Gif-sur-Yvette, France) and J. SUBIK (Vyskumy Ustav Potravinarsky, 82509, Bratislava, Czecoslavakia). Antibiotics were dissolved in ethanol and added to media at 50°.

RESULTS

Screening mutations marking the mitochondrial cyt *b* gene: The respiratory cytochrome *bc1* complex transfers electrons from ubiquinol to cytochrome *c* oxidase. Mucidin (strobilurin A) and myxothiazol are center o inhibitors preventing electron transfer at the ubiquinone redox site Qo located close to the outer side of the inner mitochondrial membrane (VON JAGOW and LINK 1986). In yeast, mitochondrial mutants resistant to myxothiazol have been mapped to specific loci in the cytochrome *b* gene. Specific amino acid substitutions in the apocytochrome *b* polypeptide at positions 129, 137, 256, and 275 led to myxothiazol resistance (DI RAGO, COPPÉE and COLSON 1989). Mitochondrial mutations conferring resistance to myxothiazol were cross-resistant to mucidin and vice versa. The molecular basis of resistance to these drugs in the photosynthetic bacterium *Rhodobacter capsulatus* is similar to that of yeast (DALDAL *et al.* 1989). Hybridization with probes specific for the yeast mitochondrial *cyt b* gene demonstrated the presence of this gene in the mitochondrial genome of *C. reinhardtii* (BOER *et al.* 1985). Accordingly, we assumed that mucidin and myxothiazol would be efficient inhibitors of mitochondrial respiration in this alga and that isolation of mutants displaying heterotrophic growth resistant to one of these inhibitors would result from alterations in the *cyt b* gene.

Preliminary experiments performed with haploid cells allowed us to isolate many spontaneous mutants displaying heterotrophic growth resistant to one of these drugs, but none were resistant to both myxo-

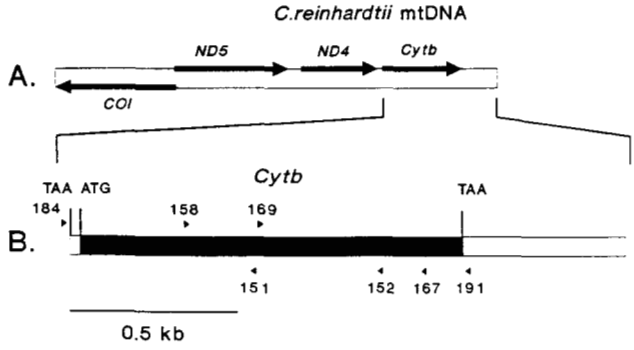

FIGURE 1.—A, Genetic map of the terminal region of the mitochondrial genome from *C. reinhardtii* (VAHRENHOLZ *et al.* 1985; BOER and GRAY, 1986; KÜCK and NEUHAUS 1986; PRATJE *et al.* 1989). B, Enlargement of the region from which the DNA sequence was determined. Arrows and numbers indicate position of oligonucleotides which were used in this investigation. ATG and TAA indicate positions of initiation and termination codons respectively. Oligonucleotides above and below the *cyt b* gene are complementary to the coding and to the non-coding DNA strand respectively. Abbreviations: *COI*, subunit I of the cytochrome oxidase; *ND4* and *ND5*, subunits of the mitochondrial NADH dehydrogenase; *Cytb*, cytochrome *b*.

thiazol and mucidin. Genetic analysis of twenty representative mutants showed that they segregate in a Mendelian fashion and appeared to be recessive. They were thus thought to be drug permeability mutants. Taking into account the high rate of recovery of such spontaneous recessive Mendelian mutants and the fact that the induced mitochondrial mutants resistant to these drugs should segregate somatically to yield resistant isolates, we decided to screen for mutants resistant to these drugs in diploid cells. We chose to focus first on mutant diploid isolates that would grow heterotrophically on both mucidin and myxothiazol. In this way selection of recessive nuclear mutants would be avoided and only putative mitochondrial mutants recovered. Indeed four diploid colonies screened for mucidin resistance appeared to be resistant to myxothiazol as well and were studied further (DPMU1, DPMU2, DPMU3 and DPMU4).

Isolation and DNA sequence of the wild-type mitochondrial cyt *b* gene: In an initial approach to isolate *cyt b* specific sequences by DNA hybridization, we synthetized two mixed oligonucleotide probes (no. 151 and 152 as shown in MATERIALS AND METHODS and Figure 1). The oligonucleotide sequences were chosen on the basis of the cytochrome *b* amino acid sequences deduced from the *cyt b* genes encoded in mitochondrial genomes of yeast, *Zea mays* and *Oenothera* taking into account the mitochondrial codon usage from *C. reinhardtii* (BOER and GRAY 1988b). This avoided making use of a heterogeneous mixture of oligonucleotide probes. To test the utility of these probes, the two oligonucleotides were hybridized with *Hin*dIII restricted mitochondrial DNA from *C. reinhardtii*. Buoyant density centrifugation allowed the separation of mitochondrial DNA from plastid and nuclear DNA. Restriction fragment *Hin*dIII-2 (HARRIS 1989), containing one of two terminal regions of the linear mtDNA, showed extensive homology with the two mixed probes (not shown). Because of difficulties in cloning the terminal *Hin*dIII restriction fragment, various *Alu*I and *Sau*3AI fragments, as well as PCR DNA fragments were cloned, to generate a set of overlapping fragments for DNA sequence determination. For this purpose, we constructed an *Alu*I and a *Sau*3AI restriction fragment library from mitochondrial DNA, using phage vector M13p19. With oligonucleotides 151 and 152 as probes, we succeeded in isolating a 850 bp *Sau*3AI restriction fragment (position 368–1223 in Figure 2) and a 280 bp *Alu*I restriction fragment (position 285–566 in Figure 2), containing parts of the *cyt b* gene from *C. reinhardtii*. From these restriction fragments, we derived a partial sequence of the *cyt b* gene, making possible the synthesis of further oligonucleotides (Nos. 158, 167, 169). With oligonucleotide 167, we isolated a 540 bp *Alu*I restriction fragment, containing the 3′ end of the *cyt b* gene. The 5′ gene sequence was generated by the "polymerase chain reaction" methodology. Using oligonucleotide 167 and oligonucleotide 184, which is homologous to the 3′ end of the *ND4* gene (PRATJE *et al.* 1989), we were able to amplify a fragment of about 1000 bp from the Chlamydomonas mitochondrial DNA. The entire set of fragments was subcloned into sequencing vectors (see MATERIALS AND METHODS) for determination of overlapping DNA sequences. The continuous stretch of 1429 bp in Figure 2 contains an open reading frame for 381 amino acid residues, located adjacent to the *ND4* gene (Figure 1). The deduced amino acid sequence shows homology with cytochrome *b* sequences from various mitochondrial genomes (Figure 4). Considerable sequence identity was found with the corresponding proteins from plants (maize 59%, Oenothera 59%), mouse (48%) and yeast (44%).

Myxothiazol-resistant mutants contain a point mutation in the *cyt b* gene: For a molecular analysis of mutated *cyt b* genes, total DNA was isolated from the four myxothiazol resistant strains. Parts of the mutant *cyt b* genes were amplified using a pair of oligonucleotides (184 and 191). After cloning the amplified fragment into bacterial vectors, the DNA sequence of the mutant genes was determined directly using as sequencing primers the set of oligonucleotides, described in the Materials and Methods. All mutants contained an identical T → C point mutation (position 410, Figure 2), which would lead to a phenylalanine to a leucine change at position 129 of the cytochrome *b* amino acid sequence (Figure 3). This residue is located within the putative transmembrane helix III of cytochrome *b* (Figure 4). No other base

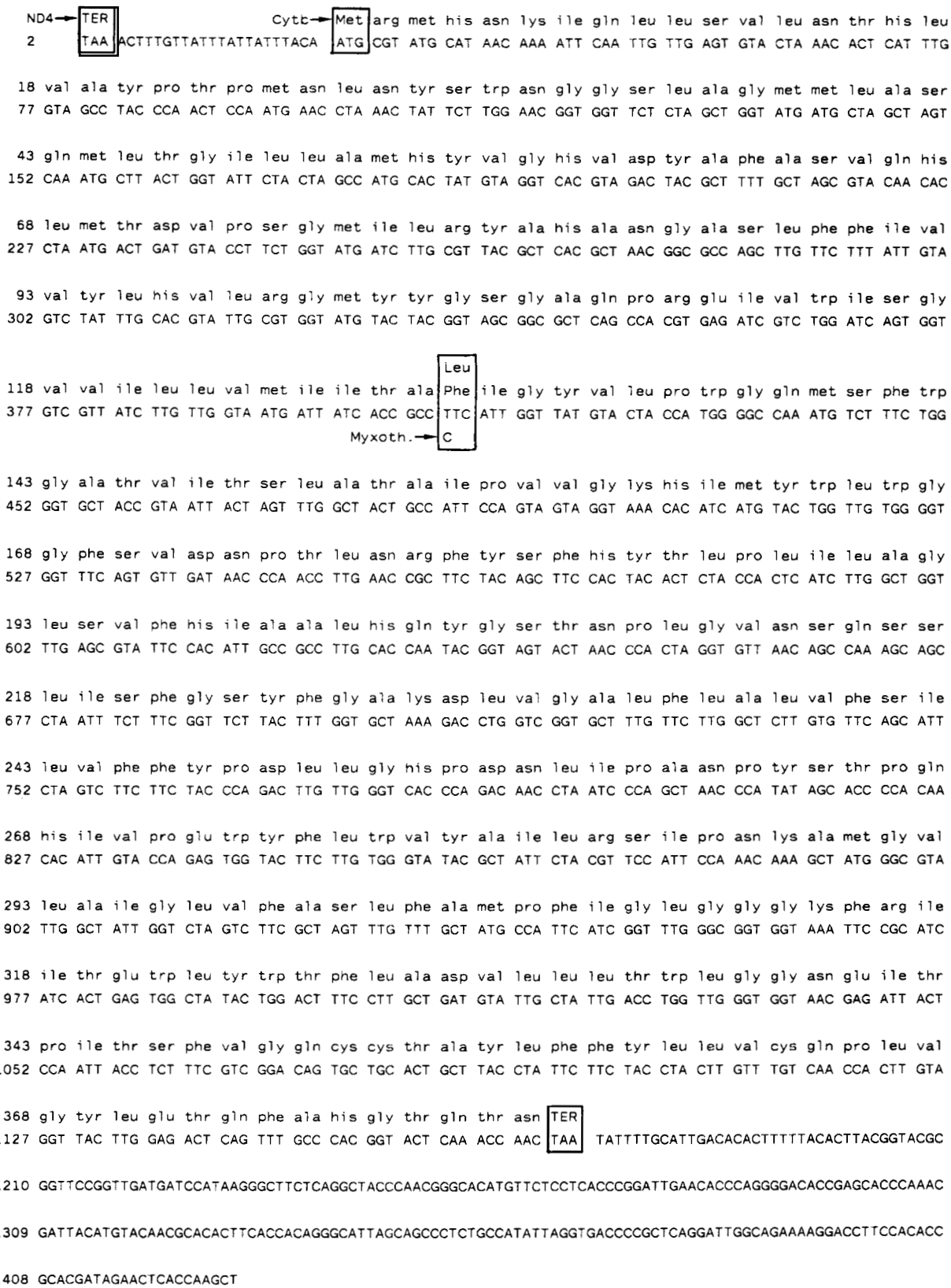

FIGURE 2.—DNA sequence of the *cyt b* gene. The non coding strand presented starts with the TAA termination codon of the *ND4* gene. Thus nucleotide no. 3 corresponds to the last nucleotide of the recently reported *ND4* DNA sequence (PRATJE *et al.*, 1989).

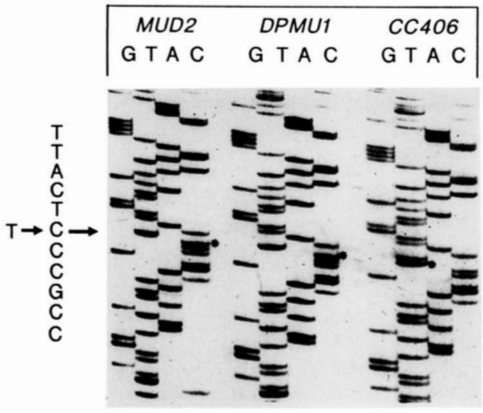

FIGURE 3.—DNA sequence of the *cyt b* gene (position 405–415) from wild-type (CC406) and mutant strains (*MUD2, DPMU1*). The asterisk denotes the nucleotide that is changed in the myxothiazol resistant mutants.

pair changes were observed in comparison to the wild-type *cyt b* gene sequence.

Genetic analysis of the MUD2 mutant: Following haploidization of the diploid DPMU2 strain, two haploid clones *MUD2 mt*$^+$ and *MUD2 mt*$^-$ were recovered which carried the same mitochondrial mutation for resistance to mucidin + myxothiazol as detected in the original diploid DPMU2 strain. Segregation analysis of *MUD2* was studied both in tetrads and in vegetative diploids on non selective and selective medium containing 2×10^{-7} M mucidin + 10^{-6} M myxothiazol.

Tetrad analysis was carried out on reciprocal crosses as depicted in Table 1. A strict uniparental minus (UP$^-$) mode of inheritance was observed in different crosses involving more than 100 tetrads from zygotes matured between 4 and 14 days in darkness after mating overnight at light intensities ranging form 1000 to 4000 lux. Vegetative diploids were recovered from various crosses and analyzed as reported in Tables 2 and 3: large variations in the frequencies of wild-type and *MUD2* progeny were observed from one experiment to the next (Table 2); however, differences in light intensity and the presence or absence of acetate had little effect on these variations (Table 2). It is not known how many copies of the mitochondrial genome carrying the resistance mutation are required for biparental heteroplasmic colonies to grow on the selective medium used. For these reasons, we will summarize our data qualitatively as follows: whereas meiotic zygotes show a pure UP$^-$ pattern of transmission of *MUD2* (Table 1) vegetative diploids transmit *MUD2* biparentally (Table 2). However, a strong but variable bias is observed for the UP$^-$ mitochondrial marker in these biparental diploid progeny (Table 2). In a given cross, the transmission of *MUD2* is highly variable from one diploid clone to the next (Table 3). Mitotic segregation of *MUD2* is observed to yield homoplasmic clones (cross III c′ analyzed in Table 2 and in Table 3).

DISCUSSION

In this paper, the amino acid changes encoding myxothiazol resistance in four independently isolated Chlamydomonas mutants were identified by sequencing their *cyt b* genes. To our knowledge, this is the first description of mitochondrial antibiotic resistant mutants in a photoautotrophic organism. All four mutants contain a single base pair change at the phenylalanine codon 129, which corresponds to a phylogenetically conserved amino acid residue of mitochondrial and bacterial cytochrome *b* proteins. Identical phenylalanine to leucine replacements have previously been described in the *cyt b* gene sequence from myxothiazol resistant strains from yeast (DI RAGO, COPPÉE and COLSON 1989) and from resistant strains of the photosynthetic bacterium *R. capsulatus* (DALDAL *et al.* 1989). Remarkably, cytochrome b_6 proteins from chloroplasts, which are phylogenetically related to mitochondrial cytochrome *b* proteins, contain a Phe-Gly-Val sequence at position 127–129 instead of the Thr-Ala-Phe sequence of mitochondrial cytochrome *b* proteins (Figure 4). That may partially explain why myxothiazol does not affect the chloroplast cytochrome b_6/f complex.

Our general conclusion from the comparative analysis is that those five domains of about 20 amino acid residues, which are conserved in all mitochondrial and bacterial cytochrome *b* sequences as well as in chloroplast b_6 proteins (HOWELL 1989), are also consistently conserved in the Chlamydomonas cytochrome *b* sequence (Figure 4). One of the most highly conserved regions (amino acid residues 125–150) of this protein is located in or close to transmembrane segment III (Figure 4). From molecular and genetic analysis of *cyt b* mutants with resistance to antibiotics, this region was hypothesized to constitute part of the Qo reaction center of cytochrome *b* (DI RAGO and COLSON 1988; HOWELL and GILBERT 1988; DALDAL *et al.* 1989). In addition, comparison of the primary amino acid sequences implies that several natural substitutions in the Chlamydomonas cytochrome *b* sequence may confer resistance or increased sensitivity to various inhibitors. This conclusion stems from analysis of mutated strains of other organisms, where homologous residues are responsible for resistance or increased sensitivity against antibiotics. For example, the Qz region (spanning amino acids isoleucine 125 to threonine 148) contains an isoleucine residue at position 125, which is only found in mitochondrial cytochrome *b* sequences from Chlamydomonas, yeast, and maize: in bacteria, methionine to isoleucine substitution at this position provides moderate levels of resistance to myxothiazol and stigmatellin (DALDAL *et al.* 1989). The reverse may be predicted from the occurrence of a glycine residue at position 232: increased sensitivity against HQNO (2-heptyl-4-hydro-

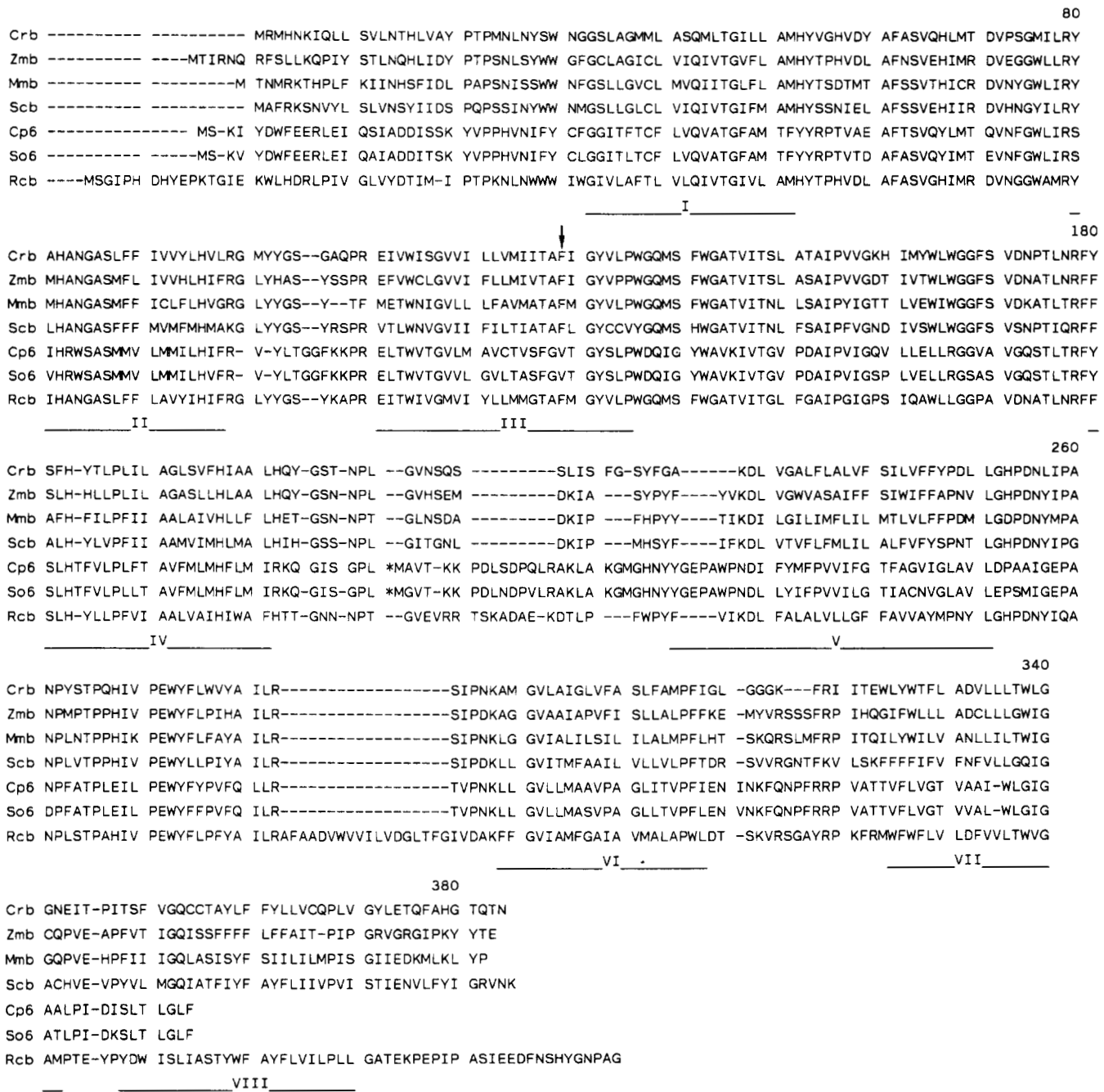

FIGURE 4.—Comparative alignment of amino acid sequences derived from mitochondrial and bacterial *cyt b* genes and chloroplast *pet B* and *pet D* genes. Roman numeral and bars indicate eight putative hydrophobic transmembrane segments of the cytochrome *b* protein (WIDGER *et al.* 1984; HOWELL, 1989). The asterisk denotes the first residue of the subunit IV polypeptide of the cytochrome b_6/f complex from chloroplasts. The arrow marks the phenylalanine residue which is changed in the myxothiazol resistant mutants. The alignment and numbering of the amino acid sequences were done according to HOWELL (1989). Data for *Chlorella protothecoides* were taken from REIMANN and KÜCK (1989). Abbreviations: Crb, Zmb, Mmb, Scb and Rcb, *cyt b* sequence from *C. reinhardtii, Zea mays,* mouse, yeast and *R. capsulatus,* respectively; Cp6, So6: sequences from cytochrome b_6 and subunit IV of the cytochrome b_6/f complex from *C. protothecoides* and from spinach, respectively.

quinoline-*N*-oxide) was observed in yeast through a threonine to glycine substitution at position 232 (DI RAGO and COLSON 1988).

Genetic analysis of a resistance mutation within the mitochondrial *cyt b* gene of *C. reinhardtii* reveals a strict UP^- mode of inheritance in meiotic progeny whereas chloroplast genes are inherited in a UP^+ manner. This results in each sexual generation of *C. reinhardtii* yielding cells having a mitochondrial genome from one parent and a chloroplast genome from the other parent. Whether this situation has some biological significance or is a result of chance is an open question. In contrast, interspecific crosses between the sibling species *Chlamydomonas eugametos* and

TABLE 1

Segregation analysis of MUD2 in tetrads

	Cross		Days of zygote maturation in darkness	Light intensity (lux) during mating and zygote formation	No. of tetrads analyzed	No. of resistant (R) and sensitive (S) clones per tetrad
A)	*MUD2*					
×	+	mt^+				
		mt^-	4	4000	12	$O^R : 4^S$
			14	4000	12	$O^R : 4^S$
A′)	+	mt^+				
×	*MUD2*	mt^-	4	4000	12	$4^R : O^S$
			14	4000	12	$4^R : O^S$
B)	*MUD2* +	mt^+				
×	+ *F22*	mt^-	8	1000	8	$O^R : 4^S$
				4000	8	$O^R : 4^S$
B′)	+ *F22*	mt^+				
×	*MUD2* +	mt^-	8	1000	8	$4^R : O^S$
				4000	8	$4^R : O^S$
C)	*MUD2* +	mt^+	8	4000	16	$O^R : 4^S$
×	+ *arg-2*	mt^-				
C′)	+ *arg-2*	mt^+				
×	*MUD2* +	mt^-	8	4000	18	$4^R : O^S$

Zygospore colonies were picked from nonselective plates and drop-tested on selective and non-selective medium to analyze the transmission of *MUD2* through meiosis. Each cross gave rise to only one type of tetrad. A 2:2 segregation of mating type was observed in all cases and the Mendelian segregation of *F22* and *arg-2* was controlled in crosses B, B′ and C, C′ respectively.

TABLE 2

Segregation analysis of MUD2 in vegetative diploids

	Type of cross[a]				Conditions for mating and selecting diploid clones[b]	Percentage growing on selective medium: Diploid clones	Percentage growing on selective medium: Subclones of a mixture of 25 diploid clones
I)	*MUD2*	*arg-2*	+	mt^+			
×					Acetate, 1000 lux	—	37
	+	+	*arg-7*	mt^-			
II)	*MUD2*	*F22*	+	mt^+			
×							
	+	+	*F31*	mt^-	a	0	0
					b — minimal, 4000 lux	35	23
					c	20	4
					c′ minimal, 1000 lux	44	14
					d acetate, 4000 lux	20	5
					d′ acetate, 1000 lux	44	8
III)	+	+	*F31*	mt^+			
×							
	MUD2	*F22*	+	mt^-	a	94	79
					b — minimal, 4000 lux	100	60
					c	60	15
					c′ minimal, 1000 lux	100	80
					d acetate, 4000 lux	100	90
					d′ acetate, 1000 lux	94	56

Twenty five diploid clones picked from non selective plates for each cross were mixed and subcloned to estimate the percentage of subclones growing heterotrophically on mucidin + myxothiazol. Individual diploid clones picked from non selective plates were drop-tested on selective and non selective medium to estimate the percentage of diploid clones growing heterotrophically on mucidin + myxothiazol.

[a] Crosses c,c′ and d,d′ were performed with the same batch of gametes.

[b] HS minimal and TAP acetate media were used.

TABLE 3

Segregation analysis of MUD2 in vegetative diploids

Type of cross (from Table 2)	Diploid clone No.	Percentage of subclones growing on selective medium
I	1	32
	2	100
	3	100
IIIc′	1	85
	2	97
	3	98
	4	100
	5	100
	6	67
	7	76
	8	65
	9	83
	10	60
	11	80
	12	0

Individual diploid clones picked from non selective plates in crosses I and III c′ as defined in Table 2 were subcloned to estimate the percentage of subclones growing heterotrophically on mucidin + myxothiazol.

Chlamydomonas moewusii, which are distantly related to *C. reinhardtii,* show the same UP^+ pattern of transmission for both mitochondrial and chloroplast genes in meiotic progeny (LEE *et al.* 1990). Strict uniparental inheritance of organelle genes means exlusion of sex for these organelles in meiotic progeny. However, most of the genetic information related to organelles lies within the nucleus and as such is subjected to recombination at each sexual generation. The complex mechanisms resulting in uniparental inheritance were proposed to have evolved to avoid insertion of foreign DNA into organelle genomes (COLEMAN 1982). The strict UP^- mode of inheritance of *MUD2* in meiotic progeny was the same as observed for the mitochondrial genome in tetrads derived from crosses between *C. smithii* and *C. reinhardtii* (BOYNTON *et al.* 1987). Random spore analysis of crosses of the *dum-1* deletion mutant with wild-type shows a UP^- mode of inheritance of this mutation but in a less stringent way (MATAGNE *et al.* 1989).

In typical crosses, a few percent of mated pairs do not form hard walled zygospores that subsequently undergo meiosis. Instead they divide soon after mating to produce clones of vegetative cells. We observed that in diploid cells, the mechanisms leading to uniparental inheritance of mitochondrial genes are not fully active. A large proportion of the colonies arising express mitochondrial markers from both gametic parents, although a strong but variable bias is seen for markers inherited from the mt^- parent. These markers segregate somatically in mitotic divisions and eventually subclones are recovered that are homoplasmic for one or the other marker type. In vegetative diploids selected from reciprocal crosses carrying the *C. reinhardtii* and *C. smithii* mitochondrial genomes, the same inheritance pattern was observed (BOYNTON *et al.* 1987). The fate of chloroplast genes in vegetative diploids is similar, although in that case a bias is observed for chloroplast markers from the mt^+ parent (GILLHAM 1969). Recombination of chloroplast genes occurs in these diploids and we hope to determine whether recombination of mitochondrial markers also takes place.

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