CHARACTERIZATION OF TWENTY-ONE MUTANTS RESISTANT TO HIGH LEVELS OF STREPTOMYCIN IN *CHLAMYDOMONAS REINHARDI*

by

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ABSTRACT

Twenty-one mutants of Chlamydomonas reinhardi resistant to high levels of streptomycin (500 µg/ml) were characterized with regard to mixotrophic growth rates, growth on solid medium containing streptomycin, organelle level of resistance to streptomycin, and pattern of inheritance of streptomycin resistance. The mutations are all uniparentally inherited as determined by zygote clone analysis, and both the chloroplasts and mitochondria may have streptomycin resistant protein synthesizing systems. Two mutants are distinct from the other nineteen on the basis of a significantly lower mixotrophic exponential growth rate. One of these slow growers, sd-12DB, is a partially streptomycin dependent mutant (maximum growth at 100 µg/ml streptomycin) and streptomycin dependence is not dominant in the plus mating type during zygote germination. Sd-12DB is a new mutation which is unique from any previously reported streptomycin dependent mutant of Chlamydomonas reinhardi. Analysis of dihydrostreptomycin binding to ribosomes was performed using crude ribosomal pellets and ribosomes fractionated on isokinetic sucrose density gradients. sd-12DB and sr-18DB failed to bind the antibiotic whereas the crude pellets (3:1) and 30S ribosomal

subunits (6:1) from wild type cells showed significant antibiotic binding, relative to these two mutants.

INTRODUCTION AND LITERATURE REVIEW

Chloroplasts contain their own genetic information but many of this organelle's proteins have been shown to be coded for by genes in the nuclear chromosomes. A microbial system uniquely suited to the identification of the specific chloroplast genes that condition chloroplast response to inhibitors of 70s chloroplast ribosome function is the unicellular green algae Chlamydomonas reinhardi. The phenotype of many uniparentally inherited chloroplast mutations previously isolated in Chlamydomonas has been resistant to or dependent on antibacterial antibiotics, including streptomycin. Sager (1954) initially isolated two types of streptomycin resistant mutants. One mutant was a nuclear mutation resistant to 100 µg/ml streptomycin and inherited in a Mendelian fashion while the other, sm2, was inherited uniparentally and resistant to high levels of streptomycin (500 µg/ml). In Escherichia coli, mutations from streptomycin sensitivity to streptomycin resistance, or dependence, results from the alteration of the ribosomal protein S12 of the small subunit (30s) of the 70s ribosome (Birge and Kurland, 1969; Ozaki, Mizushima, and Nomura, 1969). In Chlamydomonas reinhardi an altered protein has been identified in the small subunit of the chloroplast ribosome of the streptomycin resistant mutant sm2 which may be an

analogous protein to S12 (Ohta, Sager, and Inouye, 1975). Chlamydomonas reihardi, as a microbial system for the investigation of organelle genetics and biochemistry of organelle protein synthesis, is reviewed here with special emphasis upon organelle resistance to the antibacterial antibiotic inhibitors of 70s ribosome protein synthesizing functions.

Cytoplasmic Structure

Chlamydomonas reinhardi is a facultative phototroph and may be grown either phototrophically or heterotrophically utilizing acetate as a preferred carbon source (Sager and Granick, 1953). The cellular compartmentalization of these functions strongly resembles that of higher plants. The alga contains a single large chloroplast occupying one-half to two-thirds of the total cell volume. The chloroplasts of light and dark grown cells both contain chlorophyll, lamellar membrances, and numerous ribosomes in the stroma. Individual regions of DNA have been cytochemically distinguished within the chloroplast stroma (Ris and Plaut, 1962). Mitochondria are present in both light and dark grown cells as well, but they appear more fully developed in dark grown cells. Mitochondrial ribosomes are presumed to be present, but they have not been directly demonstrated by electron microscopic examination (Sager, 1972). The mitochondrion of Chlamydomonas is

assumed to contain DNA but it has not been visually demonstrated by feulgan staining in the cell (Sager, 1972). However, what is believed to be mitochondrial DNA has been isolated from enriched mitochondrial preparations (Ryan et al., 1973).

Genetic Organization

Chlamydomonas reinhardi possesses Mendelian and non-Mendelian systems of inheritance. Sixteen Mendelian linkage groups containing numerous genetic markers (Hastings et al., 1965; Sager, 1972) are located in the nuclear chromosomes (McVitte and Davies, 1971) and are characterized by a 2:2 segregation of genes at meiosis (Gillham, 1969; Sager, 1972). In contrast, cytoplasmic genes are characterized by a 4:0 segregation of genes at meiosis with all the progeny exhibiting the phenotype of the plus mating type (mt^+) parent (Gillham, 1969, 1974; Sager, 1954, 1972). This pattern of non-Mendelian segregation is termed uniparental inheritance. The most probable genetic elements responsible for uniparental inheritance are the chloroplast and mitochondrial DNAs.

Since Sager's (1954) initial recognition of the system of uniparental inheritance system of Chlamydomonas as observed in mutations resistant to high levels of the antibacterial antibiotic streptomycin, numerous additional uniparental mutants have been isolated. The majority of

these mutants are resistant to other antibiotics known to affect 70s ribosomes (Gillham, 1969, 1974; Surzycki and Gillham, 1971; Mets and Bogorad, 1971; Sager, 1972; Conde et al., 1975). Although such mutants are characterized by a 4:0 segregation of maternal (mt^+) to paternal (mt^-) markers at meiosis in reciprocal crosses, the actual distribution of markers can be more complex (Gillham, 1969, 1974; Sager, 1972). Maternal zygotes, whose meiotic products conform to the uniparental pattern of transmission, predominate in instances of cytoplasmic inheritance in Chlamydomonas but exceptional zygotes are observed in less than 1% of the matings depending upon the strain (Gillham, 1969, 1974; Sager, 1972). Paternal markers are found in the metiotic products of these exceptional zygotes. Two classes of exceptional zygotes are found. The most common class of exceptional zygote is the biparental zygote in which the meiotic products retain cytoplasmic genes from both mating type parents. The rarest class of exceptional zygote is the paternal zygote which gives rise to meiotic products bearing only the cytoplasmic markers of the mt^- parent (Gillham, 1969, 1974; Sager, 1972).

Biparental zygotes have proved to be an important instrument for the mapping of the uniparental genome of Chlamydomonas reinhardi (Sager and Ramanis, 1965, 1967, 1968, 1970, 1976a, 1976b; Sager, 1972; Gillham, 1965, 1974). As an aid in these analyses of recombination, the proportion

of biparental zygotes may be artificially increased to levels approaching 50% by (1) UV irradiation of the mt^+ parent immediately prior to mating (Sager and Ramanis, 1967), (2) by the nuclear genes mat-1 and mat-2 which act to increase (mat-1) or decrease (mat-2) the frequence of exceptional zygotes (Sager and Ramanis, 1974), and (3) by treatment of the gametes prior to mating with certain anti-metabolites (Sager and Ramanis, 1973). The cytoplasmic location of the uniparental genes may be either the chloroplast or the mitochondrion and each possibility has been extensively investigated by genetic as well as molecular approaches.

Molecular Approaches to Explain the Mechanism of Uniparental Inheritance

The classical explanation of the mechanism of uniparental inheritance in higher plants has been the disproportionate distribution of these organelles resulting from the unequal contribution of cytoplasm to the fertilized egg of the male and female parents. *Chlamydomonas reinhardi* is an isogamous heterothallic species of algae wherein the uniting gametes appear to be identical in size, and the cytoplasm of both gametes fuses completely during zygote formation. Thus, cytoplasmic exclusion does not explain maternal inheritance in *Chlamydomonas* (Sager, 1972). The DNA of *Chlamydomonas* has been intensively investigated to

elucidate an alternative mechanism of preferential exclusion.

CsCl gradients of total cell DNA from Chlamydomonas reinhardi generally show three DNAs of different buoyant densities; an α-band (nuclear DNA), a β-band (chloroplast DNA), and a γ-band (mitochondrial DNA) (Sager and Ishida, 1963; Sueoka, Chiang, and Kates, 1967; Sager, 1972; Gillham, 1974). Direct examination of these DNAs, facilitated by ^{15}N isotopic density labeling of one mating type parent, showed that in the sexual reproduction cycle of Chlamydomonas the nuclear DNA (α-band) replicates only once (semiconservatively) prior to the first meiotic division during zygospore germination (Sueoka et al., 1967). When chloroplast DNA is analyzed in reciprocal crosses between parents with DNAs of contrasting buoyant densities, the DNA of the mt^+ parent is retained in the zygote while the mt^- parent DNA disappears within the first six hours of zygote formation. The mt^+ parent DNA which persists, at the same time, undergoes a slight density shift (Sager and Lane, 1969, 1972; Schlanger and Sager, 1974a). Presuming that the observed density shift represents methylation of the mt^+ parent DNA, it has been proposed that the uniparental inheritance of chloroplast genes may result from a modification-restriction enzyme system which selectively degrades the unmethylated, and therefore unprotected, mt^- parent chloroplast DNA (Sager and Lane, 1972; Schlanger and

Sager, 1974a; Sager and Kitchin, 1975). Chiang (1968, 1971) demonstrated that the DNA having the isotopic density label of the mt^- parent was not lost but retained in the zygote and proposed that the density shift, as it was observed in his experiments, resulted from extensive recombination in the chloroplast DNA. Since Chiang's measurements were made at a later stage in the sexual cycle of Chlamydomonas, the DNA isotopically labeled as the mt^- parent may have actually represented the recycled products of its degradation (Sager, 1972), or possibly the recycled products of degraded ribosomes (Gillham, 1974). The exact fate of the chloroplast DNA has not been satisfactorily resolved in the results of these investigators but Sager's interpretations (Sager and Lane, 1969, 1972; Schlanger and Sager, 1974a) of the molecular data provide a plausible explanation of the genetic experiments.

Genetic Approaches to Explain the Mechanism of Uniparental Inheritance

The results described above are consistent if the non-chromosomal genes reside in the chloroplast DNA, but until a pattern of inheritance is determined for the mitochondrial DNA of Chlamydomonas, neither cytoplasmic DNA can be definitely declared to be the location of the uniparental linkage group. Streptolydigin (300 µg/ml), acriflavin (10 µg/ml), and ethidium bromide (3 µg/ml) prevent the synthesis of the mitochondrial membrane polypeptide P16 in Y1 mutants

of Chlamydomonas reinhardi, which is believed to be synthesized on the mitochondrial ribosomes, without inhibiting cytoplasmic or chloroplast protein synthesis (Stegeman and Hoober, 1973). These inhibitors have been employed in an attempt to induce genuine mitochondrial mutations.

When wildtype (wt) cells of Chlamydomonas are treated with acriflavin, lethal minute mutants result. They are able to divide mitotically eight to nine times under phototropic growth conditions (light with CO_2 as the sole carbon source) (Alexander, Gillham, and Boynton, 1974). The mode of cytoplasmic inheritance of the minute mutation provides the necessary contrast to the typical uniparentally inherited mutations which have been more characterized and whose genes are probably located in the chloroplast DNA. Minute mutants exhibit non-Mendelian biparental inheritance rather than non-Mendelian uniparental inheritance (Alexander et al., 1974). Alexander et al. (1974) regard the ratios seen in the exceptional zygotes recovered from this experiment as an indication that the minute mutation is transmitted to all four meiotic products independent of the mating type. Without molecular data on the fate of mitochondrial DNA during the sexual cycle of Chlamydomonas reinhardi, it can be assumed that the pattern of inheritance exhibited by the minute mutation is the pattern of inheritance of the mitochondrial genome.

Nevertheless, relying upon one or more of the pleiotropic effects of certain uniparentally inherited mutations, attempts were made prior to the publication of the above data to implicate mutation of the mitochondrial DNA as the basis of another uniparentally inherited mutation (Behn and Arnold, 1973). The ultrastructure of the mitochondrion and chloroplast of Chlamydomonas following antibiotic withdrawal from two antibiotic dependent mutants was observed in order to determine the phenotypic response of each organelle (Behn and Arnold, 1973). The uniparental mutants used in this study were dependent upon streptomycin (SD) or neamine (ND) which kill wild type cells of Chlamydomonas reinhardi under phototrophic (see page 2), heterotrophic (in the dark with acetate included as a carbon source in the medium), or mixotrophic (in the light with acetate included as a carbon source in the medium) conditions. This implies that sensitivity of wild type cells may result from inhibition of protein synthesis in the chloroplast, mitochondrion, or both (Flaks, Cox, and White, 1962; Wilkie, Saunders, and Linnane, 1967). Withdrawal of neamine, from the neamine dependent mutant, results in visible damage to the chloroplast after two days growth but no apparent alterations in mitochondrial ultrastructure were visible until five to seven days had elapsed. When the experiment was repeated with the SD mutant, mitochondrial defects were visible after two to three days growth in the

absence of streptomycin. The chloroplast becomes degenerate after four days growth in the antibiotic free medium. The conclusion drawn from these results was that the ND gene resides in the chloroplast DNA and the SD gene in the mitochondrial DNA (Behn and Arnold, 1973).

Another example of an attempt to locate a uniparentally inherited gene in the mitochondrial genome is the measurement of the frequency at which streptomycin sensitive revertants (SS) of the above SD mutant reform clones of streptomycin-dependent cells. When the SD mutant is cultured in an antibiotic free medium, sensitive revertants accumulate at a frequency of 10^{-6} to 10^{-7}. In other words, SD cells revert to SS cells at a frequency of one in a million to one in ten million, or at the expected frequency for a spontaneous mutation. The frequency of a second alteration occurring in the streptomycin sensitive revertants which reconfers streptomycin dependence when an SS revertant clone is cultured in streptomycin medium is initially 10^{-2}. This reversion frequency is ten thousand times greater than the frequency of spontaneous mutation. The authors suggest that while the SS revertant has many copies of the SS gene, it retains a few copies of the SD determinant of the original parent culture. The explanation of these frequencies with the apparent presence of many copies of this gene suggests to Schimmer and Arnold that the gene must reside in the mitochondria rather than the chloroplast since there are

many mitochondria and only a single chloroplast in Chlamydomonas (Schimmer and Arnold, 1969, 1970; Preer, 1971). However, it has been suggested that, like yeast, Chlamydomonas contains a single large branched mitochondrion (Arnold et al., 1972; Hoffman and Avers, 1973).

From the previous discussions, it becomes apparent that, without actual knowledge of the metabolic alterations occurring to organelle DNA during the sexual cycle of Chlamydomonas, those mutations inherited in a non-Mendelian fashion are most logically assumed to be located in the chloroplast DNA if they are inherited uniparentally or in the mitochondrial DNA if inherited biparentally in reciprocal crosses. Antibiotic resistance mutations to antibacterial antibiotics which inhibit 70s ribosomes have been used in phenotypic response experiments to reveal the fundamental reason why such experiments cannot be used to prove that a mutant gene is located in the chloroplast or mitochondrial DNA. The non-Mendelian uniparental mutations to antibacterial antibiotics in Chlamydomonas include: resistance to streptomycin, carbomycin, oleandomycin, spiramycin, cleocin, spectinomycin, neamine, and kanamycin; dependence upon streptomycin, spectinomycin, neamine; and conditional streptomycin dependence (Gillham, 1965; Surzycki and Gillham, 1971; Sager, 1972). These mutants are considered by some to arise from changes in the chloroplast DNA (Sager and Ramanis, 1970) but others contend that

some of the uniparentally inherited drug resistant mutations may have a mitochondrial origin as discussed above (see page

Surzycki and Gillham (1971) have adopted a set of growth conditions designed to reveal the organelle level of phenotypic response employing Mendelian and non-Mendelian (uniparental) mutants to antibacterial antibiotics. The mutants were tested to determine which protein synthesizing system, chloroplast or mitochondrial ribosomes, is resistant to the antibiotic. The rationale for this classification system is based on the assumption that either the chloroplast or the mitochondrial ribosomes may be resistant to inhibition by antibiotics. Furthermore, it was assumed that normal ribosome function in either organelle would permit normal growth under conditions which require either chloroplast or mitochondria to provide the sole source electron-transport generated ATP for cell growth. The cultures were grown phototrophically, heterotrophically, and mixotrophically in the presence or absence of selected levels of the appropriate antibiotics.

In this system, Class I mutations have a resistant chloroplast protein synthesizing system and are recognizable by an inability to grow in the presence of antibiotic under heterotrophic conditions. Class II mutations are unable to grow under photosynthetic conditions in the presence of antibiotic and have a resistant mitochondrial

protein synthesizing system. Mutants which are able to grow in the presence of antibiotic under all three conditions (phototrophic, heterotrophic, and mixotrophic) are Class III mutations and both organelle protein synthesizing systems are antibiotic resistant (Table 1). Surzycki and Gillham (1971) found that two Mendelian spectinomycin resistant mutants grow only under heterotrophic and mixotrophic conditions in the presence of spectinomycin and are Class II mutations. The remaining mutants studied are all Class III mutations (Surzycki and Gillham, 1971). On the basis of these responses, Surzycki and Gillham (1971) conclude that the uniparentally inherited antibiotic mutations in Class III are resistant in both the mitochondrial and chloroplast protein synthesizing systems.

Table 1. Growth conditions employed in the presence of antibiotic to determine organelle level phenotypic response of Chlamydomonas reinhardi mutants resistant to antibacterial drugs.[a]

	Growth conditions			
	Photo-trophic	Hetero-trophic	Mixo-trophic	Resistant Organelle
Class I	+	-	+	Chloroplast
Class II	-	+	+	Mitochondrion
Class III	+	+	+	Both

[a]Derived from Surzycki and Gillham (1971).

In order to locate the site of transcription of the genetic information responsible for the phenotypic responses observed in the above experiments, it was assumed that chloroplast DNA transcription is selectively inhibited by rifampicin, since Chlamydomonas reinhardi cells are killed by rifampicin only under phototrophic conditions. Any mutation which requires transcription of chloroplast DNA to exhibit the drug resistant phenotype should display a sensitive phenotype to the appropriate antibacterial antibiotic when grown in the presence of rifampicin. If a chloroplast DNA transcriptional product is not required, the mutant retains its characteristic resistant phenotype. All Class III mutants having Mendelian patterns of inheritance displayed a drug resistant phenotype when grown in the presence of rifamicin. Of the uniparental mutants, all were sensitive except for a single spectinomycin resistant mutant (Surzyki and Gillham, 1971). It is probable that all of the Class III uniparental mutants carry the information for their antibiotic resistances in the chloroplast DNA (Surzycki and Gillham, 1971). Later experiments on this same exceptional uniparental spectinomycin resistant mutant studied by Surzyki and Gillham (1971) showed that the mutant had spectinomycin resistant chloroplast ribosomes (Boynton et al., 1973; Burton, 1972). More recent studies, designed to explain the apparent role of both chloroplast and mitochondria in the phenotypic response of the uniparental

spectinomycin resistant mutant in these experiments, suggest that a transcriptional product of the chloroplast DNA may code for a protein that confers drug resistance upon the mitochondrial (Boynton et al., 1973) as well as the chloroplast protein synthesizing systems (Surzycki and Gillham, 1971).

The Class II and Class III antibacterial antibiotic mutants described above have undergone a detailed genetic and biochemical analysis. Class III streptomycin and spectinomycin resistant mutants, which display drug sensitive phenotypes in the presence of rifampicin, have been shown to have a drug sensitive (streptomycin or spectinomycin) chloroplast protein synthesizing system but a resistant mitochondrial protein synthesizing system (Conde et al., 1975). Conde et al. (1975) suggest that these mutants result when a chloroplast gene codes for a protein common to both the chloroplast and mitochondrial ribosomes.

With the possibility that a mutation in one organelle's DNA can affect the phenotype of both chloroplasts and mitochondria, the localization of the uniparental linkage group on the basis of any phenotypic response for a particular mutant would appear to be unreliable. The pattern of biparental inheritance for genuine mitochondrial based mutations (see page 4) and the pattern of uniparental inheritance for mutations originating in the chloroplast DNA seen in reciprocal crosses are the

only systems currently available to distinguish between cytoplasmically inherited mutations of mitochondrial and chloroplast DNA.

Ribosome Studies

Algal mutants resistant to antibiotics are not only useful in elucidating the mechanisms of uniparental inheritance, they also provide insights on the structure and function of organelle ribosomes. Mitochondrial ribosomes have not been isolated and purified for *in vitro* analysis but many mutants in *Chlamydomonas reinhardi* have been shown to have an altered chloroplast ribosomal phenotype.

Cells of *Chlamydomonas* contain cytoplasmic 80s and chloroplastic 70s ribosomes in a ratio of 3:1 in the cell (Chua, Blobel, and Siekevitz, 1973; Sager and Hamilton, 1967). Mutations to inhibitors of 70s ribosome function have been of paramount importance in studying the formation and structure-function relationships of ribosomes in bacteria. Alterations in the individual protein components of the separate subunits have been confirmed for a variety of antibiotic inhibitors employing bacterial systems. Since *Chlamydomonas* 70s ribosomes respond to these antibiotics in the same way as bacterial ribosomes, the procaryotic ribosome can be used as a model upon which to base experiments using *Chlamydomonas* chloroplast ribosomes.

The localization of the site antibiotic action in ribosome subunits of Chlamydomonas has provided results closely resembling those found with bacterial ribosomes (Davies and Nomura, 1972). The methods, first employed in bacterial systems, which have been applied to chloroplast ribosomes in Chlamydomonas are the binding of radioactive antibiotics to ribosomes (Kaji and Tanaka, 1968; Taubman et al., 1966; Bollen et al., 1969; Chang and Flaks, 1972), cross resistance to other antibiotics (Gale et al., 1972), and the construction of hybrid ribosomes by subunit exchange (Cox, White, and Flaks, 1964; Chang, Siddhikol, and Weisblum, 1969). Schlanger and Sager (1974b) have localized the uniparentally inherited resistance to five antibiotics to specific subunits of the chloroplast ribosome in Chlamydomonas. Their experiments examined cross resistance between mutants and subunit exchanges (between sensitive and resistant strains) evaluated by the activity of hybrid ribosomes in a poly-(U) directed phenylalanine incorporating system. In this manner, they have localized resistance to streptomycin, neamine, and spectinomycin to the 30s subunit. Cleocin and carbomycin mutants were shown to have resistant 50s subunits (Schlanger and Sager, 1974b; Schlanger, Sager, and Ramanis, 1972). The binding of radioactively labeled antibiotics to isolated subunits has localized the resistance of spectinomycin (Burton, 1972; Boynton et al., 1973), and streptomycin (Boschetti and

Bogdonov, 1973) to the 30s subunit and erythromycin to the 50s subunity (Mets and Bogorad, 1971). The results obtained in these experiments with Chlamydomonas coicide precisely, i.e., same antibiotic-same subunit, with those obtained for bacterial ribosomes.

Bacterial antibiotic resistant mutants have been shown to have one or more altered ribosomal proteins as detected by the methods of polyacrylamide gel electrophoresis, ribosome reconstitution experiments, and carboxymethyl cellulose or phosphocellulose column chromatography. It has been shown that, in bacteria, streptomycin resistant and streptomycin dependent mutants have an altered S12 ribosomal protein (Birge and Kurland, 1969; Ozaki et al., 1969; Isono, 1974; Itoh et al., 1975; Wittmann and Apirion, 1975) in the small (30s) subunit. Mutants which suppress streptomycin dependence have been determined to possess an altered S4 (Kreider and Brownstein, 1974; Deusser et al., 1970) or S5 (Kreider and Brownstein, 1972; Itoh and Wittmann, 1973) protein. Streptomycin dependent mutants revert to mutants which may grow in the absence of streptomycin or other amino-glycoside antibiotic. The revertants may be either sensitive or resistant to streptomycin and are called streptomycin independent mutants. Streptomycin independent mutants may be recultured in streptomycin to induce new mutations to streptomycin dependence or resistance, but, in every type of streptomycin mutant described here no

ribosomal proteins other than S4, S5, or S12 are found to be altered (Wittmann and Apirion, 1975). The S5 protein has also been identified as being responsible for spectinomycin resistance (Funatsu, Nierhaus, and Wittmann-Liebold, 1972). In E. coli and E. freundii, resistance to erythromycin is the result of an altered L8 (50s subunit) protein (Otaka and others, 1970, 1971).

Positive identification of mutant chloroplast ribosomal proteins in Chlamydomonas reinhardi has been limited to streptomycin and erythromycin resistant mutants. Ohta et al. (1975) co-chromatographed, on carboxymethylcellulose columns, differentially labeled (^{14}C and ^{3}H arginine) ribosomal proteins from a wild type strain and a selected antibiotic resistant mutant. Neamine resistant, erythromycin resistant, carbamycin resistant, and two streptomycin resistant mutants (sm2 and sr-643) were analyzed by this method and in only one of the streptomycin resistant mutants (sm2) was an altered protein detected. Two dimensional polyacrylamide gel electrophoresis of chloroplast ribosomal proteins has proven to be a more powerful tool in Chlamydomonas. The chloroplast ribosomal proteins of four Mendelian erythromycin resistant mutants (termed the ery-M1 group) were subjected to one and two dimensional polyacrylamide gel electrophoresis. The LC6 protein from the large subunit of the chloroplast ribosome of each mutant

was found to be different from its homologous protein in the wild type (Hanson et al., 1974).

Purpose of This Investigation

The large number and wide variety of streptomycin mutations which have been shown to alter the bacterial ribosome have been sufficient to define a ribosomal protein neighborhood whose physical reality has been confirmed by studies employing protein crosslinking reagents (Sommer and Traut, 1976). The difficulty in defining a homologous neighborhood in the small subunit of the chloroplast ribosome of Chlamydomonas reinhardi is partially the result of the limited variety of streptomycin mutants which have been characterized.

Twenty mutants of Chlamydomonas reinhardi, resistant to high levels (500 μg/ml) of streptomycin, have been isolated in this laboratory. The purpose of this investigation is to make use of physiological, genetic, and biochemical methods to hopefully determine if different classes can be defined among those twenty streptomycin resistant mutants. The goal of these studies is to select several mutants which might be expected to different alterations of chloroplast ribosomal proteins, and to ultimately identify and characterize the gene product (i.e., ribosomal protein) or products responsible for streptomycin resistant phenotypes in Chlamydomonas.

MATERIALS AND METHODS

Culture Techniques

Trace Element Solution

Mix 1 of the trace element solution was prepared as shown in Table 2. The next step was to dissolve 50 gm of EDTA in 250 ml of d.d. H_2O. The EDTA solution was heated to boiling and cooled to 70°C. At the same time, Mix 1 was brought to 100°C and cooled to 70°C. The two solutions were combined with constant stirring at 70°C. The combined solutions should turn green.

Mix 2 (Mix 1 + EDTA) was brought to boiling and cooled to 70°C and maintained at this temperature. It was then brought to pH 6.7 by the addition of 80-90 ml of warm (70°C) 20% (w/v) KOH using a pH meter which had been standardized with buffer warmed to 70°C. Mix 2 was then made up to a final volume of 1 liter by the addition of d.d. H_2O and placed in a cotton stoppered two liter flask. Mix 2 remained in this flask at room temperature with constant stirring until it turned purple (1 to 1-1/2 weeks).

The final dark purple trace element solution was suction filtered four times through three layers of Whatman No. 1 filter paper to remove the rust brown precipitate.

Table 2. Preparation of Mix 1 for trace element solution for Chlamydomonas culture media.[a]

Mix 1		
Salt	Weight	Final volume[b]
$ZnSO_4 \cdot 7H_2O$	22.0 gm	100 ml
HBO_3	11.4 gm	200 ml
$MnCl_2 \cdot 4H_2O$	5.06 gm	50 ml
$FeSO_4 \cdot 7H_2O$	4.99 gm	50 ml
$CoCl_2 \cdot 6H_2O$	1.61 gm	50 ml
$CuSO_4 \cdot 5H_2O$	1.57 gm	50 ml
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10 gm	50 ml
	Total volume	550 ml

[a]Derived from Surzycki (1971).

[b]Each salt is dissolved separately in the indicated volume of H_2O before any salt solution is mixed together.

This clear solution was then millipore filtered (pore size = 0.45μ) and stored in the cold (Surzycki, 1971).

Culture Media and Conditions

The stock solutions for the preparation of all culture media used are shown in Table 3. In the preparation of Beiheringck's No. 1 and No. 2, the $CaCl_2 \cdot 2H_2O$ must be dissolved separately in 75 ml d.d. H_2O to prevent the formation of a white precipitate.

Recipes for the various media employed are shown in Table 4. HSA was routinely used for the maintenance of stock cultures. The other media shown in Table 4 were used in specific instances which are detailed in later sections of the MATERIALS AND METHODS. These recipes are derived from Sueoka et al. (1967).

Strain and Isolation of Streptomycin Resistant Mutants

Wild type cultures of *Chlamydomonas reinhardi* (strain 137c) were obtained from W. T. Ebersold, University of California, Los Angeles.

Cultures of wild type cells were grown to mid log phase and plated in a lawn on minimal medium (Levine and Ebersold, 1958) containing 500 μg/ml streptomycin by Ms. Lisa Keppel. Green colonies which appeared on these plates were isolated as streptomycin resistant mutants. These

Table 3. Stock solutions for Chlamydomonas reinhardi culture media.[a]

Beijerinck's No. 1	PO_4 Buffer No. 1	Beijerinck's No. 2	PO_4 Buffer No. 2
1.0 gm NH_4NO_3	14.34 gm K_2HPO_4	10.0 gm NH_4Cl	28.80 gm K_2HPO_4
0.4 gm K_2HPO_4	7.26 gm KH_2PO_4	0.4 gm $MgSO_4 \cdot 7H_2O$	14.40 gm KH_2PO_4
0.4 gm $MgSO_4 \cdot 7H_2O$	100 ml H_2O	0.2 gm $CaCl_2 \cdot 2H_2O$	100 ml H_2O
0.2 gm $CaCl_2 \cdot 2H_2O$	pH 6.8	100 ml H_2O	pH 6.8
100 ml H_2O			

[a]Derived from Sueoka et al. (1967).

Table 4. Recipes for the preparation of Chlamydomonas media.[a]

Stock solutions	Chalmydomonas media				
	HS	HSA	YA	4%	N^-
Beijerinck's No. 1				5 ml	
Beijerinck's No. 2	5 ml	5 ml	5 ml		5 ml[b]
PO_4 Buffer No. 1				5 ml	
PO_4 Buffer No. 2	5 ml	5 ml	5 ml		5 ml
Trace Elements	1 ml	1 ml	1 ml	1 ml	
Sodium Acetate		2 gm	2 gm		2 gm
Yeast Extract			4 gm		
H_2O	990 ml	990 ml	990 ml	990 ml	990 ml
Bacto-Agar (for solid medium)	15 gm	15 gm	15 gm	40 gm	

[a]Derived from Sueoka et al. (1967).

[b]Use 5 ml of Beijerinck's No. 2 without NH_4Cl.

mutants were continuously maintained on HSA medium after transfer from the isolation medium.

Mating Analysis

Inheritance of Streptomycin Resistance

All matings to determine the pattern of inheritance of streptomycin resistance were performed as shown in Figure 1. The cultures, grown to mid log phase in HSA, were cultured under a light intensity of 4300 lux and induced to form gametes by nitrogen starvation in N^- medium. After mating the zygotes were plated on to 4% agar. The plated zygotes were exposed to chloroform to kill any remaining haploid cells for 30-45 seconds. The 4% plates were then placed in the dark for 5-6 days. At the end of this time the plates were removed and exposed to chloroform, in the same manner as above. Under a dissecting microscope, the zygotes were scraped from the 4% agar and replated on HSA. After 4 days in the light (3700 lux) the individual zygote colonies were removed and resuspended in HSA using a Lab-Line Super Mixer. The haploid cells of each zygote resuspended colony were plated separately on HSA. After 3-4 days the haploid colonies were scored for streptomycin resistance (100 µg/ml) and mating type.

In order to test mating type the haploid colonies were transferred to YA solid culture media. After 4 days growth the cells were resuspended in N^- (1 ml cotton

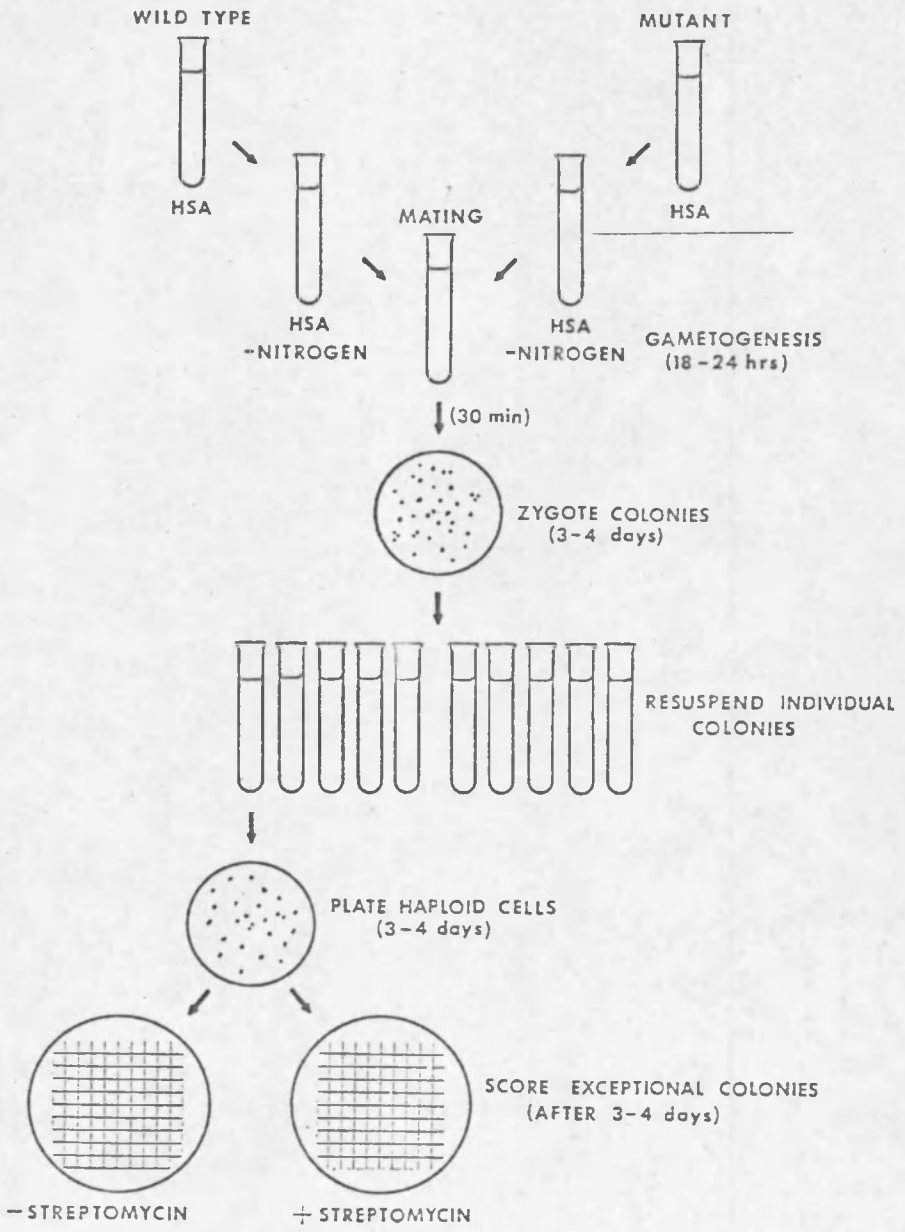

Figure 1. Analysis of the inheritance of streptomycin resistance by zygote clone analysis as modified from Conde et al. (1975).

stoppered test tubes) for 6 hours. They were then combined with wild type cells (mt^+ and mt^- separately), prepared identically, on clean glass slides separated by streaks made by a wax marking pencil. After 1 hour the cells were examined for clumping, indicating mating, under the microscope. Clumping was assumed to occur with the wild type culture of the opposite mating type (Sager and Granick, 1953; Conde et al., 1975).

UV Induction of Biparental Zygotes

When it was necessary to induce biparental zygotes in order to transfer a mutation to the opposite mating type, matings were conducted exactly as stated above with the exception that the cells of the mt^+ parent were placed under a UV lamp following gametogenesis in an uncovered sterile petri dish for 3-5 minutes. After UV irradiation, the mt^- gametes were immediately added to the UV irradiated mt^+ gametes for mating (Sager and Ramanis, 1967).

Zygote Dominance of Streptomycin Dependence

Reciprocal crosses were made and zygotes plated on 4% agar media containing streptomycin (100 µg/ml). After the dark incubation period, hemacytometer counts were made of the zygote suspensions (in HSA) from the 4% plates and equal numbers of zygotes were plated on to the HSA plates ($\pm$ 100 µg/ml streptomycin). The per cent germination was

calculated for the plates containing streptomycin and the control (no streptomycin):

$$\% \text{ germination} = \frac{\text{number of zygote colonies}}{\text{number of zygotes plated}} .$$

Streptomycin dependence is considered to be dominant in the zygote if the zygotes from a mating where the SD traid resides in the mt^+ parent require streptomycin in the medium in order to germinate (Sager and Tsubo, 1961).

Growth Curve Techniques

Absorbance Measurements of Liquid Cultures

Mixotrophic growth curves were analyzed by the method of Sorokin (1973). A sample protocol is shown in Table 5. Each mutant was inoculated into three separate 125 ml culture flasks containing 75 ml of HSA. Measurements of absorbence were made at 24 hour intervals (0-144 hours).

The exponential growth rate (R_E) for each flask was calculated from the following equation:

$$R_E = \frac{\log_2 A_{678} \text{ (72 hours)} - \log_2 A_{678} \text{ (48 hours)}}{\text{72 hours} - \text{48 hours}}$$

The exponential growth rates were averaged over the three flasks inoculated for each separate mutant culture to give a mean exponential growth rate ($\bar{R}_E$). Standard deviations (s.d.) were calculated for each mean in the following manner:

Table 5. Sample protocol for absorbance growth measurements of Chlamydomonas reinhardi.[a]

Time (hours)	Absorbance growth measurements				
	A_{678}[b]	log A_{678}	$\log_2 A_{678}$		Δ[c]
			log A_{678} x 3.32	$\log_2 A_{678}$+10	
0	0.155	-0.810	-2.869	7.311	0
1	0.196	-0.709	-2.353	7.646	0.336
2	0.258	-0.589	-1.955	8.045	0.734

[a]Table is derived from Sorokin (1973).

[b]A_{678} = the absorbance of the algal culture at a wavelength of 678 nm.

[c]Δ = the number of doublings of absorbance at the time intervals indicated from the beginning of the absorbance readings (Time = 0).

$$s.d. = \sqrt{\sum_i \frac{(R_{Ei} - \bar{R}_E)}{n - 1}}$$

where n = 3 and i = (flask 1, flask 2, and flask 3) (Steel and Torrie, 1960).

Using the statistics obtained above, Student's t was calculated for each mean according to the following equation:

$$t = \frac{R_{Ei} - \bar{R}_{Ek}}{(s.d.)\ k}$$

(Steel and Torrie, 1960). (k designates the individual mutant for which the distribution is being evaluated.) t measures the deviation of each individual R_E from the mean exponential growth rate expressed in reciprocal standard deviation units (Steel and Torrie, 1960).

Growth on Solid Medium in the Presence of Streptomycin

Each mutant and the wild type were grown in YA in 125 ml culture flasks until mid log phase. The cells were concentrated in sterile screw cap culture tubes by centrifugation at 1000 rpm for 2 minutes and washed once in HSA. The final pellets were resuspended in HSA. Using a hemacytometer, the suspensions of each culture were standardized at a concentration of 10^9 cells per ml. A nichrome inoculation needle was ground to a uniform edge resembling a screwdriver. This needle was then used to make four streaks of the resuspended cells on plates of varying

concentrations of streptomycin (0-600 µg/ml). The streak were allowed to stand in situ until the suspending medium had been absorbed by the HSA/streptomycin plate (3-4 hours). They were then placed in the light (4300 lux) and allowed to incubate for 5 days. At the end of the five day incubation period, the width of the streaks was measured, averaged over the 4 streaks per plate per mutant, and the average recorded.

Organelle Level Phenotypic Response to Streptomycin

Methods for this experiment, which tests whether chloroplast and/or mitochondria alterations are responsible for the observed uniparental phenotype, are adapted from Surzycki and Gillham (1971) as summarized in Table 1. Eight series of plates were inoculated with three mutant strains and one wild type streak per plate. Series 1 consisted of HS plates incubated in the light. Series 2 were HS plates with 100 µg/ml streptomycin which were also incubated in the light. Series 3 and 4 were duplicates of series 1 and 2, respectively, but these plates were incubated in the dark. This procedure was repeated with HSA plates taking the place of HS plates and constituted series 5, 6, 7, and 8. After 5 days incubation, the plates were inspected for cell growth.

Ribosome Analysis

Isolation of *Chlamydomonas reinhardi* Ribosomes

Cells were grown to late log phase in HSA in 2500 ml low form culture flasks. In preparation to sonication of the cells, the cultures were chilled over ice. The chilled cells were harvested using a Sorvall GSA rotor at 5600 rpm for 1 minute. The pelleted cells were resuspended in cold sonication buffer (see Tables 6 and 7). This wash was repeated two additional times after which the cells were resuspended in HS buffer at a concentration of approximately 10^9 to 10^{10} cells/ml.

The washed and resuspended cells were well chilled over ice to which NaCl had been added. While still surrounded by ice and with constant stirring, the cells are sonicated employing a Sonifier Cell Disruptor (model W-350) for 8 minutes (output control = 6; 50% duty cycle).

The sonicate was centrifuged in a Sorvall SS-34 rotor at 9100 rpm for 10 minutes. The supernatant was then centrifuged at 15,800 rpm for 45 minutes. The supernatant from this 30,000 x g spin was the S-30 fraction.

The S-30 fraction was centrifuged over a 1.5 M sucrose cushion (see Tables 6 and 7) at 40,000 rpm for 6-1/2 hours in a Beckman Ti50 rotor. The supernatant was discarded and the pelleted ribosomes were dissolved in resuspension buffer (see Tables 6 and 7). The resuspended

Table 6. Stock solutions for ribosome analysis and binding studies.[a]

Ribosome stock solutions					
Name	Chemicals	Amounts	Titrant	Final Volume	pH
Stock A	0.25 M Tris	60.5 gm	HCl	2 liters	7.5
	0.25 M KCl	37.3 gm			
	0.25 M $MgCl_2$	101.5 gm			
Stock B	0.25 M Tris	60.5 gm	HCl	2 liters	7.5
	0.25 M KCl	37.3 gm			

[a]Derived from Bourque and Wildman (1973).

Table 7. Solutions for cell disruption, ribosome preparation, and binding studies.

Ribosome analysis solutions						
Name	Chemicals	Stock A	Stock B	Titrant	Final Volume	pH
Sonication Buffer	0.25 M Sucrose 50 mM β-mercaptoethanol (7 ml)	200 ml		HCl	2 liters	7.8
Resuspension Buffer	50 mM β-mercaptoethanol (7 ml)	20 ml	180 ml		2 liters	7.5
Binding Assay Buffer		100 ml	100 ml		2 liters	7.5
Sucrose Cushion	1.5 M Sucrose 50 mM β-mercaptoethanol (0.35 ml)	10 ml			100 ml	7.5
E. coli Buffer	0.01 M $MgCl_2 \cdot 6H_2O$ (0.02 gm) 0.02 M Tris (2.42 gm) 50 mM β-mercaptoethanol (0.35 ml)			HCl	100 ml	8.0

pellets from each tube were combined and dialyzed against (2 liters) resuspension buffer for 24 hours with one buffer change (2 liters).

Isolation of Escherichia coli Ribosomes

E. coli paste (strain B) was dissolved in E. coli chilled buffer (Table 7) (2 gm cells/100 ml buffer). The cells suspended in this manner were disrupted by sonication under the same conditions used above for Chlamydomonas. The sonicate was centrifuged at 15,000 rpm for 35 minutes in an SS-34 rotor and the supernatant treated exactly as the Chlamydomonas S-30 fraction to prepare ribosomes.

Binding of Dihydrostreptomycin to Ribosomes

Binding to Crude Ribosome Pellets. D^3HSM (Amersham/Searle; 2.1 mCi/mg) was added to the dissolved crude ribosome pellets which were dialyzed against resuspension buffer containing 10 mM $MgCl_2$ (10:1 molar ratio of D^3HSM to ribosomes was used, assuming 1.0 A_{260} units is equivalent to 24 pmoles 70s ribosomes) and the mixture was allowed to incubate at room temperature for 30 minutes (Boschetti and Bogdonov, 1973). The incubation mixtures were then diluted with cold assay buffer (Table 2) and held in ice until filtration.

Millipore filters (type HA; pore size = 0.45 μm) were presoaked in cold assay buffer with care to avoid any unnecessary manipulation of the filters. A filter was applied to a sintered disk platform, attached to a suction apparatus, and gentle suction applied. The reaction mix (ribosomes and D^3HSM) was placed on top of the filter and after it had been drawn through, the filter was washed with 2 ml of assay buffer. A 15 ml column was then secured to the filtering system and the filter was washed three times with 5 ml of assay buffer (total wash = 15 ml).

The filters were then pinned to a clean, non-absorbent surface and allowed to dry thoroughly. The drying process may be assisted by placing the filters in a 60°C oven for 5-10 minutes. After drying, the filters were placed in scintillation vials and 10 ml of scintillation fluid (New England Nuclear Omnifluor, 4 gm/liter toluene) was added. The vials were then placed in a Packard Tri-Carb (3320) Liquid Scintillation Spectrometer and each vial was counted for 20 minutes. The protocol of this streptomycin binding procedure was modified from that of Kaji and Tanaka (1968).

Binding to Ribosomes Fractionated on Sucrose Gradients. Isokinetic sucrose gradients were constructed for the Beckman SW25.1 and SW 27 rotors Table 8) according to the procedures presented by McCarty, Vollmer, and

Table 8. Procedure for making isokinetic sucrose density gradients using a constant volume mixing chamber.[a]

Isokinetic gradients				
Rotor	Chamber	% Sucrose	Volume resuspension buffer	gm Sucrose
SW 25.1	mixing	10%	74.33 ml	3.82 gm
	reservoir	28.6%	150 ml	48.1 gm
SW 27	mixing	15%	225 ml	35.8 gm
	reservoir	31.6%	230 ml	82.4 gm

[a]Derived from McCarty et al. (1974).

McCarty (1974). For the SW 25.1 0.5 ml to 1.0 ml of resuspended, dialyzed ribosomes containing 10 to 20 A_{260} units were applied to a 10% to 28.6% gradient. The SW 25.1 was spun at 20,000 rpm for 15 hours and 30 minutes. Fifteen to 31.6% isokinetic sucrose gradients were used in the SW 27; 0.4 ml of sample (containing 10 to 20 A_{260} units of ribosomes) were applied to each gradient. These were spun for 12 hours at 22,300 rpm.

The gradients were monitored with a Gilford spectrophotometer and 18 drop fractions collected in Cole Scientific MC-15 centrifuge tubes with an Isco fraction collector. The individual fractions were bound to D^3HSM in the same fashion as the crude pellet ribosomal experiments described above. However, the cold assay buffer added to each gradient fraction, before incubation at room temperature in the presence of D^3HSM, was adjusted to 10 mM $MgCl_2$ by the addition of stock A to increase the $MgCl_2$ from 2.5 mM in the gradient fractions in order to optimize binding conditions.

RESULTS

Zygote Clone Analysis of the Inheritance of Streptomycin Resistance

In order to discover whether the mutants which had been isolated and shown to be resistant to high levels of streptomycin (500 µg/ml) were uniparentally inherited like other previously characterized high level streptomycin resistant mutants (Sager, 1972), zygote clone analysis was performed. Twenty-one crosses were made with wild type (streptomycin sensitive) Chlamydomonas (the reciprocal crosses were not made in this experiment), and 9846 haploid products were examined from these matings (see Table 9). Six exceptional haploid products from three different zygotes were discovered. Since 210 zygotes were tested in this experiment, the percentage of exceptional zygotes is 1.45. This percentage is consistent with previous results with uniparental mutants and it may be assumed that all 21 mutations to streptomycin resistance are uniparentally inherited.

Mixotrophic Growth Rate Analysis

As an aid in developing criterion upon which to separate the mutants into defined classes, their rate of growth in mixotrophic liquid culture (HSA) was analyzed employing absorbence measurements. The results (Table 10)

Table 9. Zygote clone analysis of the inheritance of streptomycin resistance.

Zygote clone analysis				
Cross (mt^+ x mt^-)	No. of haploid progeny tested	% mt^+	% SR	% Exceptional zygotes
sr-01 x wt	640	48.4	100	-
sr-02 x wt	576	51.4	99.7	00.3
sr-03 x wt	572	50.5	100	-
sr-04 x wt	354	47.5	100	-
sr-05 x wt	640	48.0	99.5	00.5
sr-06 x wt	512	49.6	100	-
sr-07 x wt	512	51.4	100	-
sr-010 x wt	438	45.7	100	-
sr-011 x wt	640	52.0	100	-
sr-012 x wt	300	52.3	100	-
wt x sr-11	300	51.3	00.0	-
wt x sr-12	300	53.7	00.0	-
wt x sr-13	640	47.3	00.0	-
wt x sr-14	292	58.9	00.3	00.3
wt x sr-15	300	47.3	00.0	-
wt x sr-16	512	50.8	00.0	-
wt x sr-17	438	57.1	00.0	-
wt x sr-18	300	49.0	00.0	-
wt x sr-19	300	48.7	00.0	-
wt x sr-110	640	50.3	00.0	-
wt x sd-12	640	51.7	00.0	-

Table 10. Mixotrophic exponential growth rate analysis.

Mixotrophic growth rate analysis			
	Exponential growth rate		
Mutant	R_E	s.d.	Student's t 95% C.I.
sr-01	0.041	0.003	(0.034, 0.048)
sr-02	0.101	0.002	(0.096, 0.106)
sr-03	0.102	0.001	(0.100, 0.104)
sr-04	0.110	0.004	(0.100, 0.120)
sr-05	0.085	0.003	(0.078, 0.092)
sr-06	0.090	0.002	(0.085, 0.095)
sr-07	0.091	0.007	(0.074, 0.108)
sr-010	0.097	0.001	(0.095, 0.099)
sr-012	0.095	0.003	(0.088, 0.102)
sr-11	0.092	0.018	(0.047, 0.137)
sr-12	0.109	0.003	(0.102, 0.116)
sr-13	0.118	0.001	(0.116, 0.120)
sr-14	0.110	0.004	(0.100, 0.120)
sr-15	0.105	0.002	(0.100, 0.110)
sr-16	0.096	0.007	(0.079, 0.113)
sr-17	0.089	0.003	(0.082, 0.096)
sr-18	0.079	0.006	(0.064, 0.094)
sr-19	0.074	0.001	(0.071, 0.076)
sd-12	0.062	0.001	(0.060, 0.064)
wt	0.121	0.002	(0.116, 0.126)

revealed statistically significant difference in the response of the mutants to these growth conditions. sr-01DB, sr-12DB, and sd-12DB have R_E (mean exponential growth rates) much lower than the wild type and lower than most of the other mutants.

When Student's t test was applied to these means and 95% confidence intervals calculated for the R_E, sr-01DB and sd-12DB had confidence intervals distinct from each other and from every other mutant examined (see Table 10). Figure 2 shows a visualization of the confidence intervals of the mean for a representative sample of the 21 mutants. This representation dramatizes the difference between sd-01DB and the wild type (wt). sr-01DB which is off-scale on Figure 2, has an even lower R_E than sd-12DB. These two mutants clearly grow much slower in mixotrophic culture than the wild type or the other streptomycin resistant mutants.

sr-13DB, sr-14DB, and sr-04DB appear to grow most like the wild type in these experiments while the rest of the mutants, excluding sr-11DB with a standard deviation greater than 0.005, appear to constitute a class intermediate between the wild type and the two slow growers.

Growth on Solid Medium in the Presence of Streptomycin

An additional parameter on which to base a comparative classification of these mutants which reveals

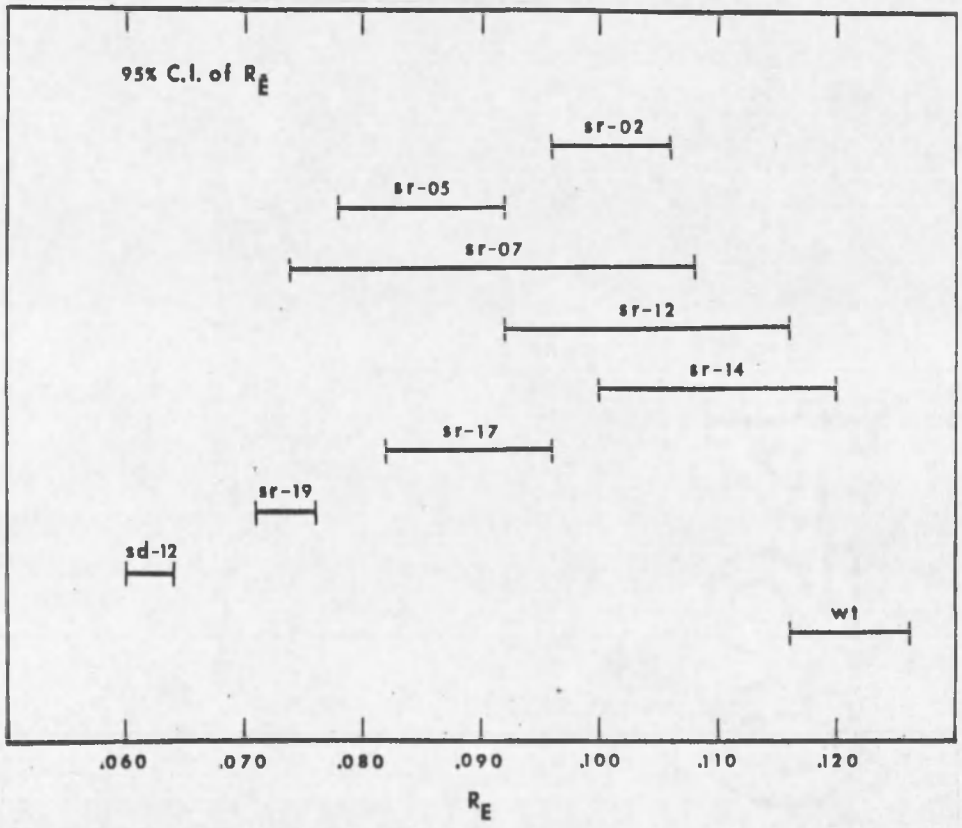

Figure 2. Visualization of the 95% C.I. of the R_E of several streptomycin resistant mutants and the wild type.

differences is growth in the presence of streptomycin on HSA plates. Wild type cells grown on solid mixotrophic media fail to produce normal green colonies at a streptomycin concentration of 50 µg/ml (Sager, 1972). This was confirmed by our experiment where the growth in the width of streaks applied to HS plates containing streptomycin was measured after 5 days (Figure 3). The response typical of all of the streptomycin resistant mutants, except sd-12DB, is represented by sr-09DB. These mutants grew consistently well over the range of streptomycin concentration employed. sd-12DB, however, shows a distinctive growth pattern which we have termed partial streptomycin dependence. There is an obvious growth maximum at a streptomycin concentration of 100 µg/ml (Figure 3). These results definitely confirm that sd-12DB differs in response to streptomycin from the other mutants.

Zygotic Dominance of Streptomycin Dependence

sd-12DB, not being obligately streptomycin dependent, resembles Sager's streptomycin dependent SM4 mutant (Sager and Tsubo, 1961), which requires streptomycin in the medium for zygospore germination when the SD mutation is carried by the mt^+ parent. My previous experiments (page 41) have shown that streptomycin is not required for zygote germination when the mutation is carried by the mt^- parent. Employing UV irradiation of the mt^+ gametes immediately

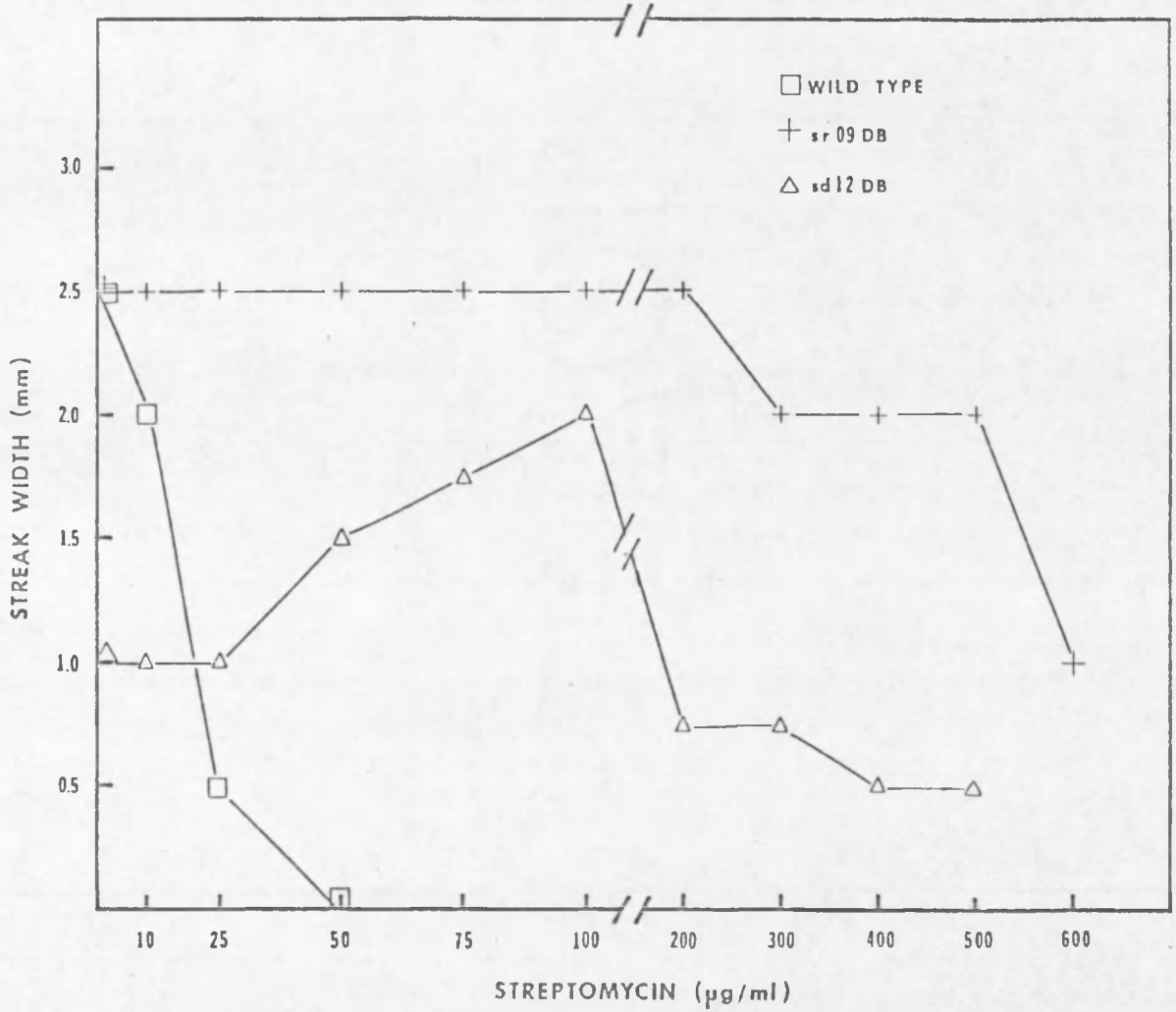

Figure 3. Growth response to varying concentrations of streptomycin on HSA plates.

prior to mating, the mutation (sd-12DB) was successfully transferred to the plus mating type. sd-12DB (mt^+) was crossed to the wild type (streptomycin sensitive) and the zygotes plated on HSA with and without streptomycin (100 µg/ml). Seventy-two per cent of the zygotes germinated on the plates containing streptomycin and 69% of the zygotes germinated on the plates without streptomycin. This is not a significant difference and it may be concluded that sd-12DB is not the same mutation as sm4 and that the sd-12DB phenotype is not dominant in the zygote.

Organelle Level of Phenotypic Response to Streptomycin

A portion of the experiments to determine the organelle level of phenotypic response to antibacterial antibiotics by Surzycki and Gillham (1971) were repeated here on solid media containing streptomycin. According to this test, mutants were found to have resistant chloroplast and mitochondrial protein synthesizing systems and are Class II mutations according to Surzycki and Gillham's scheme (see Table 1).

Binding of Dihydrostreptomycin to Ribosomes

The binding of D^3HSM to the ribosomes of *Escherichia coli* is dependent upon the concentration of Mg^{++}. At 37°C in 70 mM KCl and 50 mM Tris-HCl, the maximum binding of D^3HSM to *E. coli* ribosomes occurs at 12 mM Mg^{++} (Teraoka and

Tanaka, 1973). This experiment was repeated for Chlamydomonas crude ribosomal pellets from wild type cells in the presence of 25 mM KCl and 25 mM Tris-HCl. The results (Figure 4) show that as $MgCl_2$ concentration increases, the binding of D^3HSM increases and starts to plateau at 10 mM $MgCl_2$. Binding reaches its highest level at 15 mM $MgCl_2$. In performing further experiments using the filter assay method for the binding of D^3HSM, a concentration of 10 mM $MgCl_2$ was used.

Crude ribosome pellets from sonicated cells containing 80s and 70s, ribosomes from a typical streptomycin resistant mutant (sr-18DB), the partially streptomycin dependent mutant (sd-12DB), and the wild type were filter assayed for their ability to bind dihydrostreptomycin. Sensitive ribosomes will bind D^3HSM and resistant ribosomes will not. The results are shown in Figure 5. The wild type ribosomes, as expected, specifically bind high levels of D^3HSM while the two mutants do not. The binding ratio seen in this experiment (approximately 3.5:1) is comparable to what Kaji and Tanaka (1968) observed for E. coli ribosomes.

To determine the subunit site of resistance to streptomycin, the ribosomal subunits were separated on sucrose density gradients and gradient fractions were assayed for D^3HSM binding. E. coli ribosomes were used as a control in these experiments. E. coli (Figure 6) showed

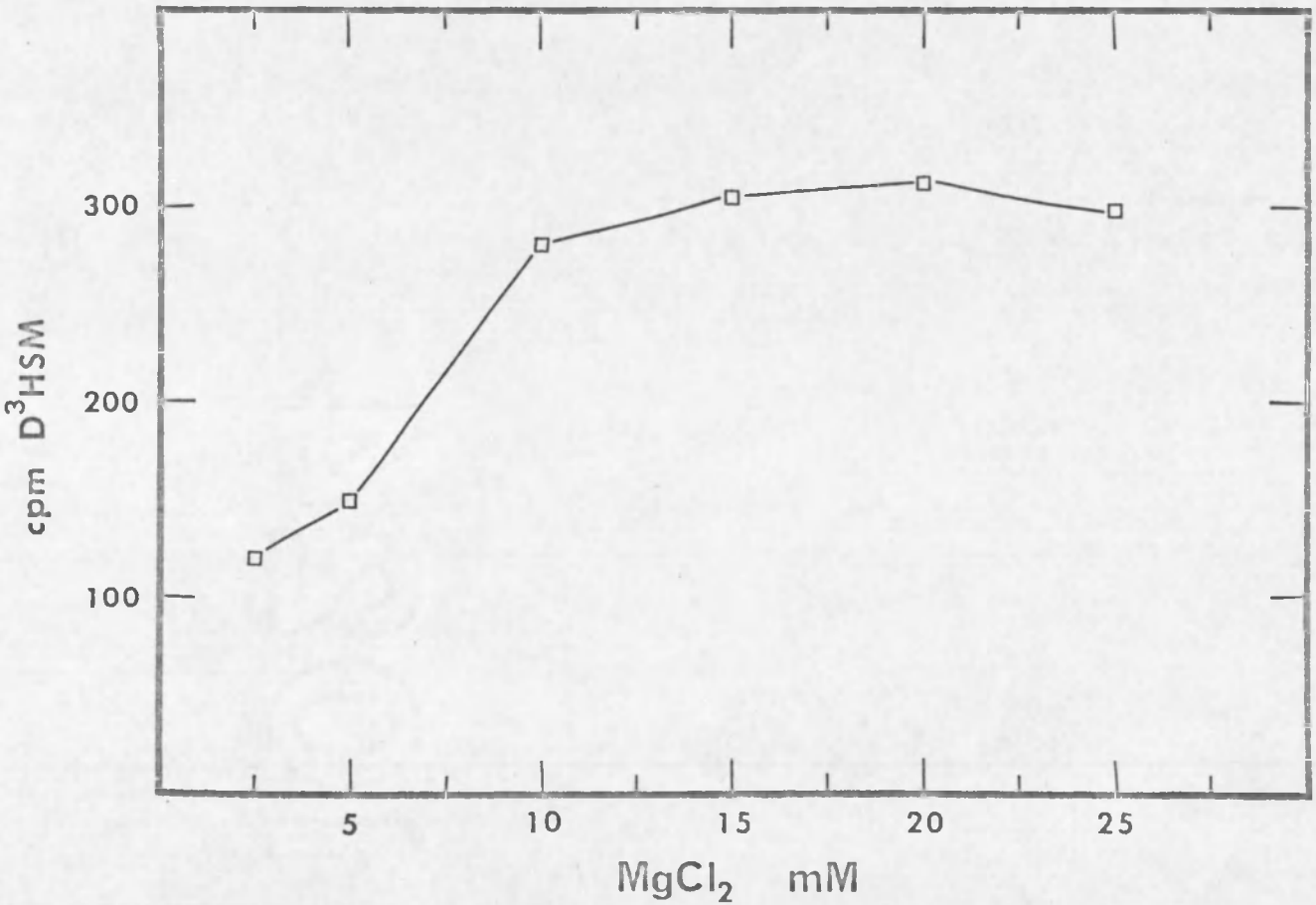

Figure 4. The effect of $MgCl_2$ concentration on the binding of D^3HSM to crude pellets.

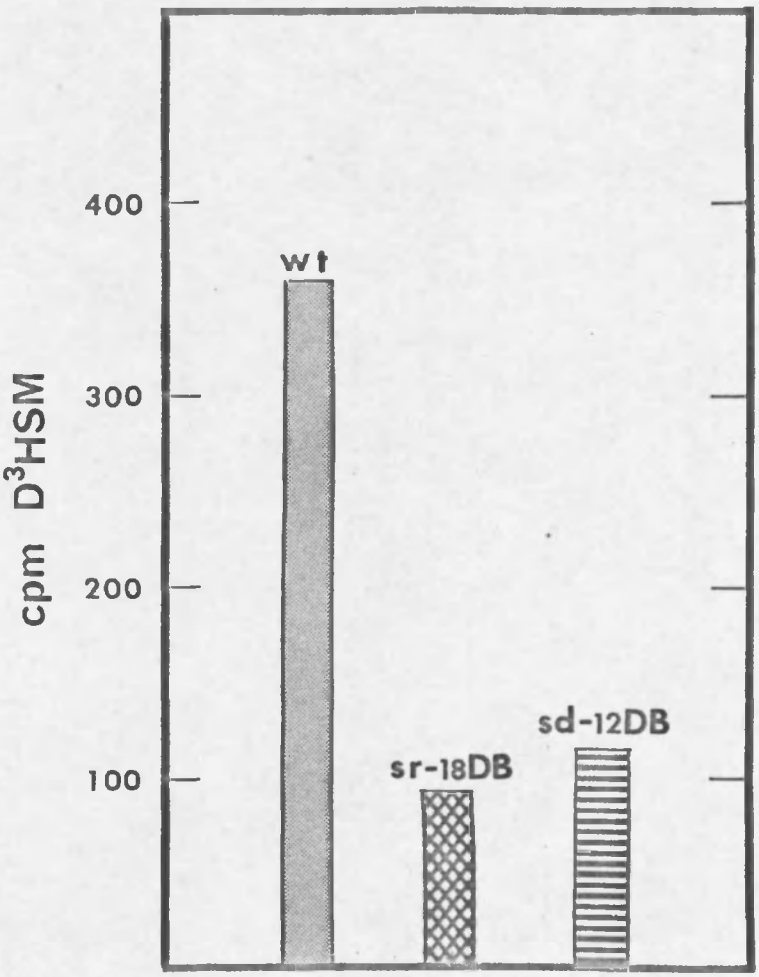

Figure 5. Binding of D^3HSM to the crude ribosome pellets of wildtype, a typical streptomycin resistant mutant (sr-18DB), and to sd-12DB in 10 mM $MgCl_2$.

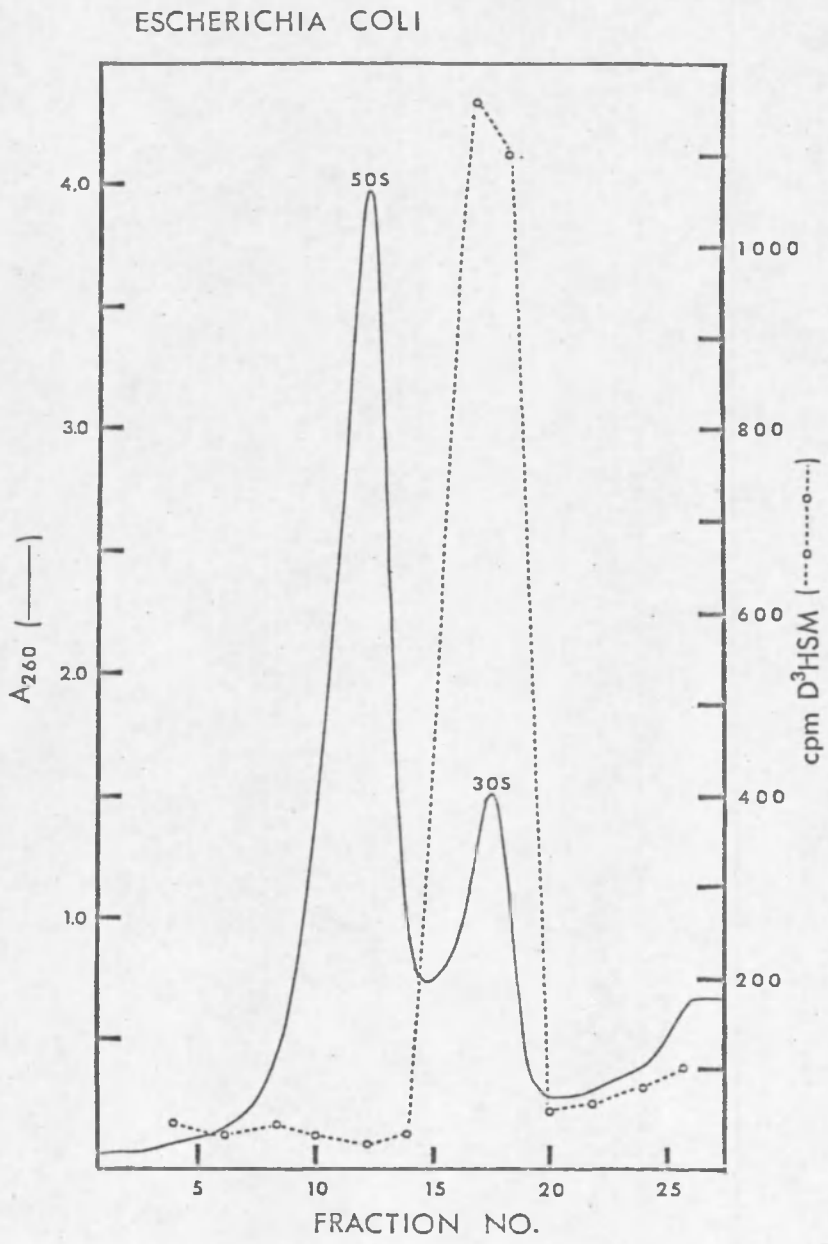

Figure 6. Binding of D^3HSM to gradient fractions of E. coli ribosome subunits SW 27 isokinetic sucrose density gradient.

excellent binding to the 30s subunit which has been shown to be the site of streptomycin resistance (Ozaki et al., 1969). When the experiment was repeated with Chlamydomonas reinhardi wild type ribosomes the results were equally good (Figure 7). Only the 30s subunit bound D^3HSM. sr-18DB (Figure 8) and sd-12DB (Figure 9) failed to bind D^3HSM. The results shown here are consistent with findings published elsewhere (Boschetti and Bogdonov, 1973).

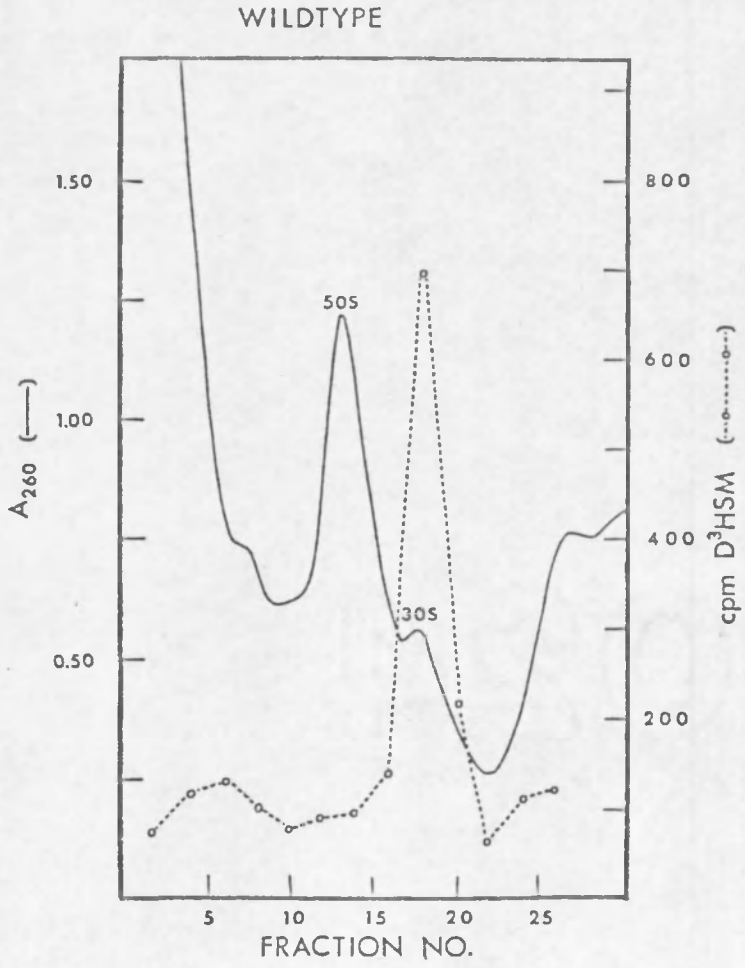

Figure 7. Binding of D^3HSM to ribosomes of wildtype Chlamydomonas reinhardi fractionated on an isokinetic SW 27 sucrose density gradient.

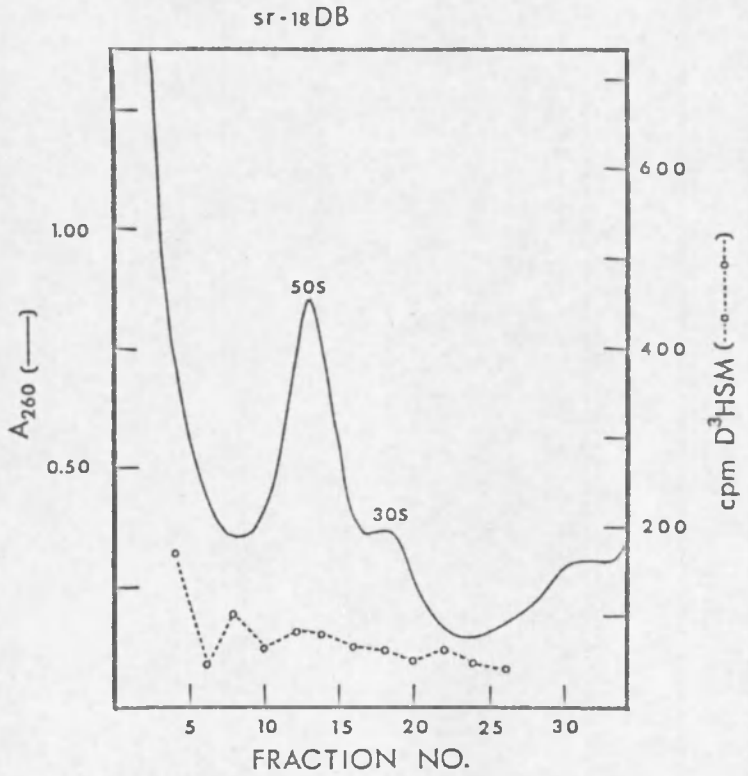

Figure 8. Binding of D^3HSM to ribosomes of a streptomycin resistant mutant (sr-18DB) of Chlamydomonas reinhardi fractionated on an isokinetic SW 25.1 sucrose density gradient.

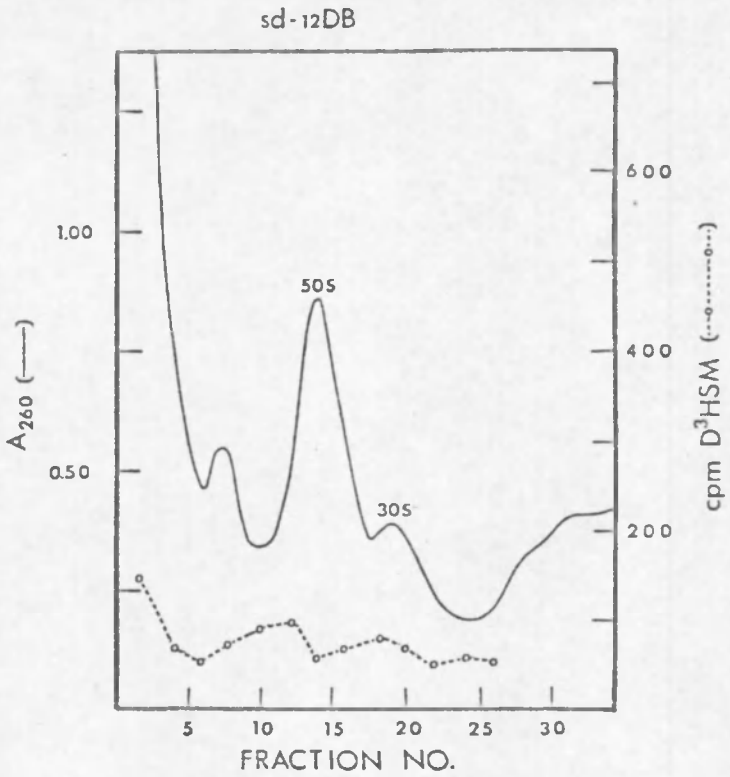

Figure 9. Binding of D^3HSM to ribosomes of the partially streptomycin dependent mutant, sd-12DB, fractionated on an isokinetic SW 25.1 sucrose density gradient.

DISCUSSION

All twenty-one mutations of the green alga Chlamydomonas reinhardi to streptomycin resistance from sensitivity are uniparentally inherited alterations. Crosses conducted by zygote clone analysis showed a 2:2 segregation pattern for the nuclear marker (mating type) and a 4:0 or 0:4 (depending upon the mating type of the streptomycin resistant mutant) segregation pattern of streptomycin resistance. Based upon this it can be concluded that all twenty-one mutations are uniparentally inherited and are presumably the result of alteration in the chloroplast genome.

Analysis of the mean exponential growth rates (R_E) revealed a wide range of response to the mixotrophic culture conditions. The R_E ranged from 0.041 for sr-01DB to 0.121 for the wild type control. Without exception, every streptomycin resistant mutant showed a slower growth rate than the wild type. However, sr-04DB, sr-11DB, sr-12DB, sr-13DB, and sr-14DB cannot be distinguished statistically from the wild type on the basis of this experiment. These five streptomycin resistant mutants have 95% C.I., calculated by Student's t-test (Steel and Torrie, 1960), which overlap the 95% C.I. determined for the wild type R_E. sr-11DB has an exceedingly large standard

deviation, however, and may not actually belong to this group of fast growing mutants. sd-12DB and sr-01DB have 95% C.I. which are mutually exclusive as well as distinctly separate from any of the other mutants. When the middle range of the growth rate response is analyzed, a large overlapping of the 95% C.I. of the mean exponential growth rates is seen. sr-02DB, sr-03DB, sr-07DB, sr-012DB, sr-11DB, sr-12DB, sr-13DB, sr-14DB, sr-15DB, sr-16DB, and sr-17DB all overlap another member of this group at some point. Only sr-11DB spans the entire range. sr-05DB, sr-06DB, sr-18DB, and sr-19DB form a cluster of slower growing mutants in the mid-range of growth response. This experiment has revealed three classes of mixotrophic growth rate response: Class I members are fast growers having an R_E equal to or greater than 0.089 and includes the majority of the mutants analyzed; Class II display intermediate growth rates and include mutants whose growth rates fall between 0.074 and 0.090 inclusive; Class III are slow growers and contain sr-01DB and sd-12DB which have R_E of 0.041 and 0.062, respectively. These classes are somewhat arbitrary and are assigned as a tentative scheme to order the results of an experiment which showed that these mutants have pleiotropic growth rate phenotypes.

When growth was analyzed on phototrophic solid medium (lacking acetate as a carbon source) in the presence of varying concentrations of streptomycin a more substantial

phenotypic difference among the mutants was observed. All of the mutants, excluding sd-12DB, showed a uniform phenotypic response at the streptomycin concentrations tested. A distinctive growth maximum, somewhat depressed from the maxima of the other mutants, was observed for sd-12DB. This pattern of response has been termed partial streptomycin dependence. On the basis of this experiment, sd-12DB may be considered to be unique from the twenty other mutants.

Since sd-12DB is not obligatorily dependent upon streptomycin, it is distinct from Gillham's (Surzycki and Gillham, 1971) sd-3-18 mutant which stringently requires 20 µg/ml streptomycin for growth in liquid culture. In order to determine if it is unique from another well characterized streptomycin dependent mutant of Chlamydomonas reinhardi, SM4 (Sager and Tsubo, 1961), sd-12DB was tested for zygotic dominance or streptomycin dependence. sm4 grows maximally at 100 µg/ml streptomycin and requires streptomycin in the germination agar for zygote germination in a cross where the mutation is carried by the mt^+ parent (Sager and Tsubo, 1961). sd-12DB does not exhibit dominance of streptomycin dependence in the zygote and is unique from Sager's sm4.

In repeating a portion of Surzycki and Gillham's (1971) experiment to determine the streptomycin resistant organelle protein synthesizing system, the mutants responded uniformly. According to Surzycki and Gillham's

criteria, all twenty-one mutants have both resistant chloroplast and mitochondrial protein synthesizing systems.

Direct examination of the ribosomal phenotype by dihydrostreptomycin binding (Kaji and Tanaka, 1968) revealed no differences in phenotype among the mutants sr-18DB and sd-12DB. sr-19DB and sr 01DB, not shown here, also displayed a resistant phenotype by failing to bind the antibiotic. Sensitivity to streptomycin was localized in the 30s subunit of the chloroplast ribosome in the wild type by this technique.

In Escherichia coli a ribosomal protein neighborhood has been defined by the numerous mutants isolated which are streptomycin resistant, streptomycin dependent, or streptomycin independent revertants from streptomycin dependence. The physical reality of this neighborhood has been confirmed by studies employing protein crosslinking reagents (Sommer and Traut, 1976). Streptomycin independent revertants show phenotypic response in terms of depressed growth rates similar to sd-12DB (Birge and Kurland, 1970). Since secondary mutations in either of the ribosomal proteins S4 or S5 enable streptomycin dependent strains of Escherichia coli to revert to streptomycin independent strains, it is proposed that the mutant sd-12DB may prove to assess multiple alterations of the chloroplast ribosome similar to the streptomycin revertants of E. coli.

SUMMARY

Twenty-one newly isolated mutations to high-level streptomycin resistance (500 µg/ml) in Chlamydomonas reinhardi were tested by zygote clone analysis for their pattern of inheritance of streptomycin resistance. In non-reciprocal crosses all segregation patterns observed were consistent with a pattern of uniparental inheritance (4:0 or 0:4 depending on the mating type of the mutant). The level of streptomycin resistance and the segregation patterns observed show that all twenty-one mutations are uniparentally inherited.

When these mutants were examined in a mixotrophic liquid culture, a wide variation in exponential growth rates was observed. Sr-01DB and sd-12DB grow considerably slower than the wild-type or any of the other streptomycin resistant mutants. Statistical analysis of the mean exponential growth rates in this experiment showed, at the 95% confidence limits, that the mean exponential growth rates of these mutants (sr-01DB and sd-12DB) were statistically significantly unique from each other and the ohter mutants examined.

Sd-12DB, when cultured on solid photographic medium in the presence of varying concentrations of streptomycin (0-600 µg/ml), showed a distinctive growth maximum at 100

µg/ml streptomycin. This pattern of partial streptomycin dependence clearly separated this mutant from the twenty others which displayed a single uniform phenotype across the streptomycin concentrations employed.

sd-12DB showed no zygotic dominance of streptomycin dependence when reciprocal crosses were made with and without streptomycin present in the zygote maturation and germination media. These results differentiated phenotypically between this mutant and Sager's sm4 (Sager and Tsubo, 1961) and confirmed the previously presumed pattern of uniparental inheritance of this mutant. sd-12DB is also unique from the only other well characterized streptomycin dependent mutant in the literature, sd-3-18, which in liquid culture is obligately streptomycin dependent requiring at least 20 µg/ml streptomycin (Surzycki and Gillham, 1971).

According to one test, the organelle level of phenotypic response to streptomycin observed suggested that all twenty-one mutants had both resistant chloroplasts and resistant mitochondria. The analysis of chloroplast ribosomes for binding of streptomycin by dihydrostreptomycin showed that the ribosomes from wild type cells strongly bind the antibiotic while chloroplast ribosomes from sd-12DB and sr-18DB do not bind dyhydrostreptomycin. The binding sites for streptomycin have been localized to the 30s chloroplast ribosomal subunit in wild type cells. However, the 30s subunits of those streptomycin resistant

mutants tested and those of the mutant sd-12DB do not bind streptomycin.

LIST OF ABBREVIATIONS

C.I.	Confidence Interval
d.d. H_2O	Distilled de-ionized water
D^3HSM	Tritiated Dihydrostreptomycin
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
4%	Four per cent agar
HSA	High Salt Acetate (mixotrophic culture)
HS	High Salt (phototrophic culture)
mt^+	Plus mating type
mt^-	Minus mating type
N^-	Minus nitrogen (gametogenesis)
poly-(U)	Poly uridylic acid
R_E	Exponential growth rate
$\bar{R}_E$	Mean exponential growth rate
s.d.	Standard deviation
SD	Streptomycin dependent
SI	Streptomycin independent
SR	Streptomycin resistant
SS	Streptomycin sensitive
UV	Ultraviolet light
wt	Wild type

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