Molecular Markers for Rapidly Identifying Candidate Genes in *Chlamydomonas reinhardtii*: *ERY1* and *ERY2* Encode Chloroplast Ribosomal Proteins

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ABSTRACT

To take advantage of available expressed sequence tags and genomic sequence, we have developed 64 PCR-based molecular markers in *Chlamydomonas reinhardtii* that map to the 17 linkage groups. These markers will allow the rapid association of a candidate gene sequence with previously identified mutations. As proof of principle, we have identified the genes encoded by the *ERY1* and *ERY2* loci. Mendelian mutations that confer resistance to erythromycin define three unlinked nuclear loci in *C. reinhardtii*. Candidate genes ribosomal protein L4 (RPL4) and L22 (RPL22) are tightly linked to the *ERY1* locus and *ERY2* locus, respectively. Genomic DNA for RPL4 from wild type and five mutant *ery1* alleles was amplified and sequenced and three different point mutations were found. Two different glycine residues (G_{102} and G_{112}) are replaced by aspartic acid and both are in the unstructured region of RPL4 that lines the peptide exit tunnel of the chloroplast ribosome. The other two alleles change a splice site acceptor site. Genomic DNA for RPL22 from wild type and three mutant *ery2* alleles was amplified and sequenced and revealed three different point mutations. Two alleles have premature stop codons and one allele changes a splice site acceptor site.

A large collection of chemically induced mutations exists in *Chlamydomonas reinhardtii* (DUTCHER 2000; HARRIS 2001). Several approaches have been used to identify the gene products of various loci. A locus of interest can be cloned by complementation (PURTON and ROCHAIX 1994; ZHANG *et al.* 1994; FUNKE *et al.* 1997), by identifying new alleles with an insertional tag (TAM and LEFEBVRE 1993), by identifying new alleles with a transposable element (SCHNELL and LEFEBVRE 1993), or by positional cloning from a nearby physical marker (DUTCHER *et al.* 2002). The availability of expressed sequence tags and genomic sequences that have mapped onto a physical/genetic map make it possible to identify candidate genes for various loci. RANUM *et al.* (1988) and VYSOTSKAIA *et al.* (2001) have developed restriction fragment polymorphism markers for use with Southern blots and single nucleotide polymorphisms, respectively. The availability of mapped molecular markers that can be scored quickly and easily should make it possible to rapidly identify mutations that correspond to a candidate sequence.

Erythromycin is an antibiotic that blocks the peptide exit tunnel of bacterial and other prokaryotic-like ribosomes (GALE *et al.* 1981; GABASHVILI *et al.* 2001). Resistance to erythromycin is conferred by mutations in ribosomal proteins L22 and L4 in bacteria. In addition, resistance is conferred by mutations in the 23S ribosomal RNA in domain V as well as by mutations in loci that encode 6-N',N'-adenosyl dimethyltransferase or dimethyladenosine transferase. The absence of methylation of 2508A in 23S rRNA confers resistance (LAI and WEISBLUM 1971). These sequences are possible candidate genes. The unicellular green alga, *C. reinhardtii*, is sensitive to erythromycin. Using radiolabeled erythromycin, METS and BOGORAD (1971) showed the site of action of erythromycin to be the chloroplast of Chlamydomonas.

Mutations in Chlamydomonas that confer resistance to erythromycin have been isolated in both nuclear and chloroplast loci. Genes for the 23S rRNA are located in the chloroplast and two mutations in the rDNA confer resistance (HARRIS *et al.* 1989; MAUL *et al.* 2002). Mutations in three nuclear loci (*ERY1, ERY2,* and *ERY3*) also confer resistance to erythromycin. These loci map to linkage groups X, XIV, and I, respectively (HANSON and BOGORAD 1977, 1978; EVES and CHIANG 1982). Alterations in the mobility of chloroplast ribosomal proteins from *ery1* and *ery2* mutant strains have been observed on one- and two-dimensional gels (METS and BOGORAD 1972; DAVIDSON *et al.* 1974).

Mutations that confer resistance to erythromycin provide proof of principle that we can identify and then map candidate sequences with respect to previously

identified mutant loci. We have identified the gene products for two of the three nuclear *ERY* loci. The *ERY1* locus encodes ribosomal protein L4 (RPL4) and the *ERY2* locus encodes ribosomal protein L22 (RPL22).

MATERIALS AND METHODS

Strains and culture media: Crosses were made between CC1952 and several laboratory strains derived from strain 137c using protocols described previously (HARRIS 1989). CC-1952 is a strain isolated from the wild that has extensive molecular polymorphisms relative to the lab strain, 137c (GROSS *et al.* 1988). Strains are listed in Table 1. Media were as described (LUX and DUTCHER 1991).

In agreement with current nomenclature rules for *C. reinhardtii*, the alleles at the *ERY1* loci are changed from *ery1a, ery1b, ery1c,* and *ery1d* to *ery1-1, ery1-2, ery1-3,* and *ery1-4*. Ery11, ery12, and ery14 (HARRIS *et al.* 1974) are named *ery1-6, ery1-7,* and *ery1-8*. The same changes have been made for *ERY2* alleles and are listed in Table 1.

PCR protocol: Primers for the mapping markers were chosen using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; ROZEN AND SKALETSKY 2000). DNA was isolated as described (JOHNSON and DUTCHER 1991). The mapping was performed in 25-µl reactions using *Taq* polymerase with 20 mM Tris, 50 mM KCl, pH 8.3 buffer with 20 pmol of each primer, 5% DMSO, 1 nM dNTP, and MgCl$_2$ concentrations as indicated in Table 2. The cycling parameters were 95° for 5 min, 95° for 1 min, at the temperature in Table 2 for 1 min, 72° for 1 min per kilobase of product length, repeated 30 times, and 72° for 10 min. Restriction digests were performed directly on the PCR products as indicated in Table 2. Products were displayed on 2.0% agarose gels with ethidium bromide.

Colony PCR: DNA from colonies from tetrads of *ery1* × CC1952 and *ery2* × CC1952 was made using the REDextract-N-AMP blood PCR kit (Sigma, St. Louis). The kit was used according to the manufacturer's directions with the exception that 20-µl reactions were used. Restriction digests were performed in the PCR mix directly.

Screening the bacterial artificial chromosome library: PCR products from genomic DNA were used to screen filters obtained from Genome Systems (now available from Clemson University Genomics Institute) using random primed labeling methods as described elsewhere (DUTCHER *et al.* 2002). Identification of additional bacterial artificial chromosome (BAC) clones was made by searching the JGI database of BAC end sequence (http://bahama.jgi-psf.org/prod/bin/chlamy/home.chlamy) using BLASTn or tBLASTn (ALTSCHUL *et al.* 1990).

Sequencing: Isolated genomic DNA from wild type and *ery1* mutant strains was amplified using primers GCACTTCGCAT TGTTTAGGT and CGTCCTCAATGATGATGTGGT or GCC AGGCCATCCTAAACTAA. Klentaq long and accurate polymerase (BARNES 1994) was used with the following conditions to amplify DNA for sequencing: 35 cycles of 1 min at 94°, 2 min at 52°, and 10 min at 68°. A final 30-min extension period at 68° was included. The product was purified from a 1% agarose gel using the gel purification kit from QIAGEN (Valencia, CA). For *ERY2*, four pairs of primers were used with Klentaq long and accurate polymerase to amplify DNA with the following conditions: 35 cycles of 1 min at 94°, 2 min at 56°, and 10 min at 68°. No gel purification was needed. The DNA was sequenced using BigDyeV.3 in conjugation with the protein and nucleic acid chemistry laboratory (Washington University School of Medicine). For *ERY1* DNA, seven forward and seven reverse primers were used for sequencing. For *ERY2*

DNA, three forward and three reverse primers were used for sequencing. The Sequencher Program (Gene Codes, Ann Arbor, MI) was used for assembling the sequence reads into contigs.

Modeling the protein structure: The protein sequences of RPL4 and RPL22 were submitted to SwissModel (http://www.expasy.org/swissmod/) to be fit to the crystal structures of the homologous proteins from *Harloarcula marismortui* (IFFK) and *Deinococcus radiodurans* (1LNR; GUEX *et al.* 1999). The predicted chloroplast signal peptide was removed for the modeling, as there was no similarity with the bacterial sequences used for crystallization. The predicted structure was examined using Deep View: Swiss-PDF viewer.

RESULTS

Physical markers for mapping: To facilitate the rapid mapping of sequence obtained from expressed sequence tags (ESTs) and genomic sequence, we have developed molecular PCR-based mapping markers that distinguish between alleles in two Chlamydomonas strains (137c and CC1952; GROSS *et al.* 1988; VYSOTSKAIA *et al.* 2001). Many of the loci were selected on the basis of their previously known map position (http://www.biology/duke.edu/chlamy_genome/) and loci within 10–15 map units of their respective centromeres were preferentially chosen. PCR products for 64 markers were amplified using the primers and conditions that are listed in supplementary material available on the web (Appendix 1 at http://www.genetics.org/supplemental/). These markers ranged in length from 200 to >1000 bp. Eleven of the 64 markers generate PCR products in the two strains that are distinguishable on 2% agarose gels. The remaining 55 markers require digestion by a restriction enzyme to produce a distinguishable marker as indicated in Appendix 2, available online at http://www.genetics.org/supplemental/.

Meiotic progeny panel: Genomic DNA was isolated (JOHNSON and DUTCHER 1991) from 172 meiotic progeny from crosses of 137c-derived strains and the polymorphic strain, CC1952 (Table 1). These DNAs were placed into 96-well microtiter plates for monitoring the segregations of the markers in Appendix 1, available online at http://www.genetics.org/supplemental/. Figure 1 shows 2.0% agarose gels for three different markers. The segregation for LC1 on linkage group II and LC5 on linkage group XIX was performed with 94 meiotic progeny and two parental strains; segregation for acetyl glutamate kinase on linkage group I was performed with 82 meiotic progeny and two parental strains. The last two lanes in the bottom right of Figure 1, A–C, contain the parental DNA. We find that 40–100% of the DNA samples from meiotic progeny produce products that can be interpreted with an average success rate of 80%. The segregation of the 62 markers was determined for up to 172 meiotic progeny; some markers were analyzed from only one of the plates of progeny. The linkage analysis is shown graphically in Figure 2.

Strain	Genotype	Source
CC1952	Wild type	Chlamydomonas Genetics Center
	apm1-100; mt+	Lux and Dutcher (1988)
	bld2-2; rgn1-1; ery3-1; mt+	This laboratory
	bld2-2; rgn2-1; mt+	This laboratory
CC-943	*ery2-2; mt+*	Chlamydomonas Genetics Center (*ery2b*)
CC-893	*ery2-4; mt+*	Chlamydomonas Genetics Center (*ery2d*)
CC-75	*ery2-5; mt+*	Chlamydomonas Genetics Center (*ery2e*)
CC-941	*ery1-1; mt−*	Chlamydomonas Genetics Center (*ery1a*)
CC-942	*ery1-2; mt−*	Chlamydomonas Genetics Center (*ery1b*)
CC-1365	*ery1-3; pf2*	Chlamydomonas Genetics Center (*ery1c*)
CC-948	*ery1-4; arg7*	Chlamydomonas Genetics Center (*ery1d*)
CC-70	*ery1-5*	Chlamydomonas Genetics Center (*ery10*)
CC-72	*ery1-6*	Chlamydomonas Genetics Center (*ery11*)
CC-77	*ery1-7*	Chlamydomonas Genetics Center (*ery14*)

(The full set of data is shown in Appendix 2 at http://www.genetics.org/supplemental/).

Mapping ESTs to candidate genetic loci: Many mutations have been identified in *C. reinhardtii*, but few of their gene products are known (Dutcher 2000; Harris 2001). We set out to determine if we could identify the gene products for several previously characterized loci using a candidate gene approach. Loci that confer resistance to erythromycin often encode ribosomal proteins in eubacteria (Spahn and Prescott 1996). Three nuclear loci that confer resistance to erythromycin in *C. reinhardtii* (*ERY1–ERY3*; Davidson *et al.* 1978) have previously been identified.

ESTs for ribosomal proteins: Resistance to erythromycin in bacteria is conferred by mutations in ribosomal proteins L4 and L22, in domain V of the 23S rDNA gene, and in methyladenosine transferase. We searched the EST database for homologs of L4 and L22 ribosomal proteins (RPL4 and RPL22) and found matches (Table 2). Bogorad and colleagues established that a single ribosomal protein was altered in some *ery1* alleles. This protein was called L6 in their numbering scheme on the basis of its mobility in two-dimensional gels. Although no equivalence to the *Escherichia coli* L6 protein was implied by their numbering (Hanson and Bogorad 1978), we found an EST for the Chlamydomonas L6 homolog and established a linked molecular marker for it as well. We identified an EST that shows similarity to a dimethyl adenosine transferase (DMAT) as well as its genomic DNA sequence (Li *et al.* 2003).

DNA from 40, 43, and 40 meiotic progeny from crosses of *ery1*, *ery2*, or *ery3* by CC1952, respectively, was obtained and used in colony PCR with primers for RPL4, RPL22, and the DMAT homolog. We were unable to generate a polymorphism for RPL6 so we used a nearby gene, BI99. We observed complete linkage between RPL4 and *ERY1* and between RPL22 and *ERY2*. BI99 and DMAT showed no linkage to any of the *ERY* loci (Appendix 2 at http://www.genetics.org/supplemental/). BI99 showed linkage to markers on linkage group IX, and DMAT showed linkage to markers on linkage group III (Appendix 2 at http://www.genetics.org/supplemental/).
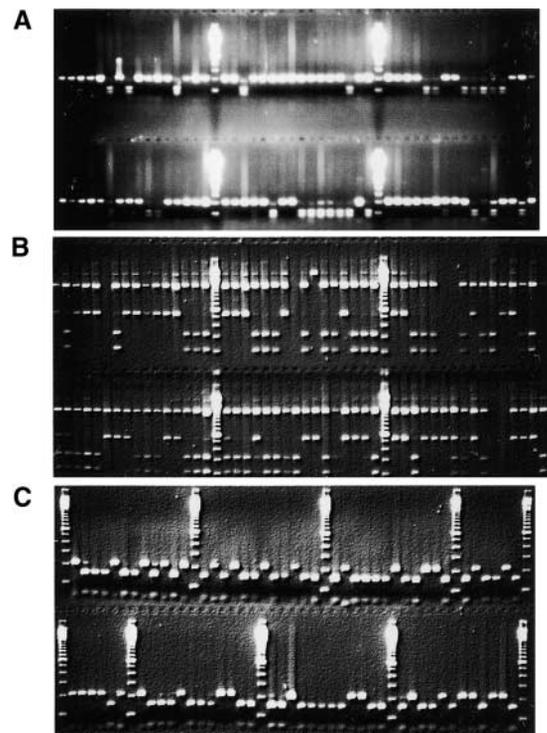

Figure 1.—Segregation of LC1, LC5, and AGK in meiotic progeny. DNA from 94 meiotic progeny was used for PCR with primers to (A) LC1, a dynein light chain; (B) LC5, a dynein light chain, and (C) AGK, acetyl gluatamate kinase. In A–C, the 137c and CC1952 parents are shown at the bottom right.
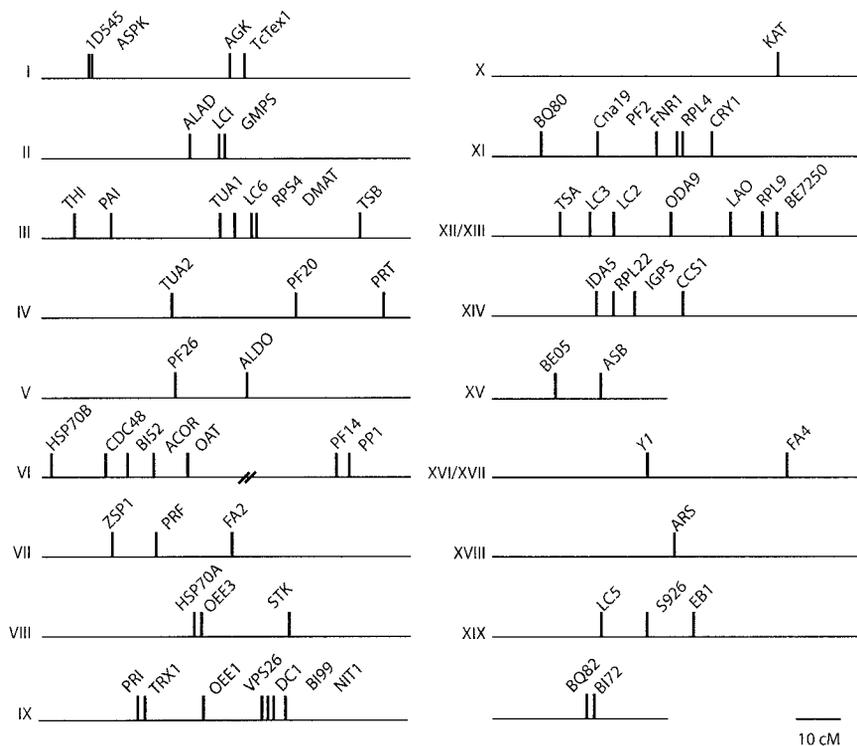
Figure 2.—Map locations of the physical markers (presented in Appendixes 1 and 2 at http://www.genetics.org/supplemental/). One set of markers could not be placed on any one of the linkage groups. This should not be considered evidence for a new linkage group, as we have not examined centromere linkage. Slash lines on linkage VI indicate that the two sides are >50 map units apart. Accession numbers for acronym entries not in ChlamyDB or Table 2 are as follows: ACOR, BI873565; AGK, BM519173; ALAD, U19876; ALDO, X69969; ARS, X52304; ASPK, AF014927; CCS1, U71000; CDC48, BU647835; Cna19, BE337032; DC1, BU-646668; EB1, BG843058; FA4, BI718037; FNR1, U10545; GP40, AF525923; HSP70A, M76725; HSP70B, X96502; KAT, AF-205377; LAO, U78797; PP1, AF156101; PRF, BQ821551; PRI, BU653640; TRX, X78822; S926, X62135; STK, AF33023; TcTex1, AF039437; VPS26, BM003137; and ZSP, S44199. Accession numbers for anonymous ESTs are BE05, BE056715; BI99, BI993933; BI52, BI528510; BQ80, BQ80-8040; and BQ82, BQ824925.

No loci that confer drug resistance have been mapped to either of these regions to date.

ERY1 encodes RPL4: Previously, seven alleles that confer resistance to erythromycin were shown to be linked to one another (Hanson and Bogorad 1977) and this locus was designated *ERY1* (Harris 1989). We sequenced 2536 bp of genomic DNA from wild type and from five of the mutant strains. The predicted Chlamydomonas protein is 243 amino acids long. It has 58% identity to the homolog in *Nicotiana tabacum* and 57% identity to the homolog in Nostoc sp. PPC7120, a cyanobacterium. The amino terminus, which is likely to be the chloroplast transit signal, shows little similarity among these three proteins (Figure 3). Two alleles, *ery1-2* and *ery1-6*, have a single nucleotide change of G to A that results in a change in the splice site from GT to AT at the end of exon 2. This change results in the loss of the restriction enzyme recognition site for *Sty*I and cosegregates with erythromycin resistance in 43 meiotic progeny for the *ery1-2* allele and in 12 meiotic progeny for *ery1-6*. The *ery1-5* allele has a single nucleotide change from G to A that results in a glycine-to-aspartic acid change at amino acid 102. This mutation results in the acquisition of an *Nru*I recognition site and cosegregates with erythromycin resistance in 10 meiotic progeny. The *ery1-3* and *ery1-7* alleles have a G-to-A mutation that results in a glycine-to-aspartic acid change at amino acid 112. This mutation results in the acquisition of a *Bcd*I recognition site and cosegregates with erythromycin resistance in 9 *ery1-7* meiotic progeny. Thus, we

observed no recombination between the *ery1* DNA polymorphisms and the erythromycin resistance phenotype. In addition, five different alleles have three different missense mutations.

ERY2 encodes RPL22: Previously, seven linked alleles were identified as conferring resistance to erythromycin (Mets and Bogorad 1972; Davidson *et al.* 1978) and this locus was designated *ERY2* (Harris 1989). We sequenced 1077 bp of genomic DNA from wild type and three of these mutant strains. The predicted Chlamydomonas protein is 171 amino acids long. It is 57% identical to the homolog from *Medicago sativa* and 55% identical to the homolog from *Porphyra purpurea*, a red alga. The *ery2-2* and *ery2-5* alleles have a G-to-T change that results in the introduction of an amber codon at amino acid 108 (Figure 4). The *ery2-4* allele has a G-to-A change resulting in an alteration in the splice site from the consensus GT to AT. If the message in *ery2-4* were not spliced, the protein would be truncated prematurely at a stop codon in the intron. These changes result in the acquisition of the restriction recognition sites for *Bfa*I and the loss of an *Hph*I recognition site for the *ery2-2* and *ery2-4* alleles, respectively.

DNA from 20 meiotic progeny from crosses of *ery2-2* × CC1952 and 20 meiotic progeny from crosses of *ery2-4* × CC1952 were analyzed for segregation of the mutant alleles with respect to the CC1952 allele. We observed no recombination between the *ery2* DNA polymorphism and the erythromycin resistance phenotype. The resistance phenotype is tightly linked to the physical marker.

<div align="center">TABLE 2</div>

<div align="center">**Chlamydomonas homologs found in ESTs and BAC library**</div>

Gene product	Arabidopsis accession number	Chlamydomonas EST accession number (ACE number)[a]	e-value of EST to Arabidopsis	Map location (linkage group)	BAC clones found by hybridization
RPL4	E84584	BI726433 (20020630.1181.1)	$5e$-19 $(2e$-47$)^b$	XI	ND
RPL22	P56795	BI875477	$3e$-23	XIV	9f11, 9e19, 15i6, 23p23
Dimethyladenosine transferase (DMAT)	BAC42078	BQ825671	$1e$-06	III	ND
Anthranilate synthase β (ASB)-chain	NP_200597	BG848432 (20021010.2520.2)	$8e$-49 $(7e$-40$)^b$	XV	28j17
Phosphoribosyl transferase (PRT)	AAB02913	BF867722	$8e$-10	IV	ND
Phosphoribosyl anthranilate isomerase (PAI)	NP_196178	5o20^c	—	III	ND
Indole 3-glycerol phosphate synthase (IGPS)	NP_199633	BE726366 (20021010.7896.1)	$1e$-27 $(1e$-86$)^b$	XIV	3m23, 39c8
Tryptophan synthase-α (TSA)	CAB77584	BG844950 (20021010.1813.1)	$6e$-50 $(5e$-84$)^b$	XII/XIII	20g13, 18i23, 32l22, 7a6, 39e4, 3i14, 21d1j, 21j4

ND, not done.

[a] The ACE number is the assembly of ESTs by the Chlamydomonas Genome Project (http://www.biology.duke.edu/chlamy_genome). These data were not available at the initiation of this work.

[b] E-value for the assembled contigs (ACE) compared to Arabidopsis homolog.

[c] Found in BAC end sequence (http://bahama.jgi-psf.org/prod/bin/chlamy/home.chlamy.cgi).

In addition, three different alleles have two different point mutations.

Resistance to other macrolide antibiotics: Tylosin and spiromycin are used extensively for treatment of animals with bacterial infections. These antibiotics, like erythromycin, bind in the narrow part of the peptide exit tunnel to occlude peptide exit (HANSEN *et al.* 2002). The side chain of spiromycin contacts RPL4 and the side chain of tylosin contacts RPL22. We asked if the mutations in *ery1* and *ery2* confer resistance to these related antibiotics. Wild-type strains are sensitive to 75 μg/ml of both compounds. The seven *ery1* alleles confer resistance to both tylosin and spiromycin from 75 to 300 μg/ml while the three *ery2* alleles confer resistance only to tylosin in the same concentration range.

<div align="center">DISCUSSION</div>

We have developed 64 PCR-based molecular markers that can be easily scored. Thirty of these are based on the data from the Chlamydomonas Genome Project that provided a framework. New markers include genes for the enzymes of the tryptophan biosynthetic pathway. We have mapped the genes for anthranilate synthase-β (ASB), phosophoribosyl transferase (PRT), anthranilate phosphoribosyl isomerase (PAI), indole 3-glycerol phos-

phate synthase (IGPS), and tryptophan synthetase-α (TSA; Figure 2; Table 2). Originally, *MAA* loci were identified by resistance to 5-methylanthranilic acid (DUTCHER *et al.* 1992; PALOMBELLA and DUTCHER 1998). Many mutations that confer resistance to 5-MAA in Arabidopsis are in genes that encode enzymes of the tryptophan biosynthetic pathway (LAST and FINK 1988). TSA maps to linkage group XII/XIII near the *MAA1* locus, IGPS maps to linkage group XIV near the *MAA4* locus, PAI maps to linkage group III, PRT maps to linkage group IV, and ASB maps to linkage group XV. Thus, the linkage between these loci and these genes provides further evidence for the efficacy of placing genomic and expressed sequence tags onto the genetic map to facilitate the identification of the genes that correspond to previously identified mutations.

We show that *ERY1* encodes ribosomal protein L4 by linkage and sequencing multiple alleles. DAVIDSON *et al.* (1978) suggested the protein encoded by the *ery1-2* allele had both a different isoelectric point and a different molecular weight from the wild-type protein. The change in the splice site acceptor site would be consistent with this observation. Under several scenarios, translation will stop prematurely in the third exon.

The $G_{102}D$ and $G_{112}D$ changes are near the alteration that confers resistance to erythromycin in *E. coli* (CHIT-

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                                     :         :. .        .              :**:
Nostoc       ------------------------------MVESVVK-----NWQGEQIGQKTFELRVAKETTAAHIVHR
Tobacco      MAASLSFFSSSIFLSNPNIQSSKHLLFRSPKQLSVAAIATIRSEVATIPILSFDGAEVGSTTLNLKSAPLDTARAVVHR
E. coli      ------------------------------MELVLK-------DAQS--ALTVSETTFGRDFNEALVHQ
Chlamydomonas -------MQTMRVAFRPAATSRSTVVTRAS---AVAAPASIPYKAA------DGSSKGTQQLALKVA-EDSAKGLVHR
             1......10.......20.......30.......40.......50.......60.......70.......80


             :.        .  *:** :  **:** *.* **: ** **.** * *     :*: .*** *..:*:*  . .:*:* * *: :.
Nostoc       LVRQQTNARQGTASTKTRAEVRGGGRKPWRQKGTGRARAGSIRSPLWRGGGVIFGPKPRDFDLKMNRKERRLALRTAFV
Tobacco      LTTDLRNQRRGTASTLTRSEVRGGGIKPYPQKKTGRARRGSNRTPLRPGGGVVFGPKPKDWSVKINKKEKRLAISTALS
E. coli      VVAYAAGARQGTRAQKTRAEVTGSGKKPWRQKGTGRARSGSIKSPIWRSGGVTFAARPQDHSQKVNKKMYRGALKSILS
Chlamydomonas LVMVQQNARQGTASTLTRSEVRGGGKKPYAQKGTGNARRGSSVSPLFPGGGVTFGPKPKDWSISMNKKERRLALATALQ
             ........90......100.......110.......120.......130......140......150.......160
                                                    D                            ery1-5
             _____                                                              ery1-3
                                                    D                            ery1-7
                                                    D                        +   ery1-2
                                                                             +   ery1-6


             *:**.:  ..  .*** : :  : .:          *:: :  :: *:.**: .: :  . :. .:: :
Nostoc       RI--DDLILVEEFSNELSRPKTKDLVAAFTRWGAEPES-KILLILSEFPENVYLSARNIENLKLIAADQLNVYDLLHAD
Tobacco      AS--ENTIVVEEFNDKFEKPKTKEFIDLMRRWGLDPKE-KSLFLMTDVSDNVILSSRNIGTLKMLTPRTLNLFDILDSE
E. coli      LVRQDRLIVVEKFS--VEAPKTKLLAQKLKDMALED----VLIITGELDENLFLAARNLHKVDVRDATGIDPVSLIAFD
Chlamydomonas AT--ADMIVVESLAGKLQDTKTKSMVALLEKLGANAMERKVLLITKEERPDVTLAGRNIAKLTMNTASAISVFDVLNAD
             ........170.......180.......190.......200.......210.......220.......230.......240


             :::  .*:  ::.
Nostoc       IVVTTSALEKIQEVYNG-----------------------------
Tobacco      LVLTKSAVDFLNERYGDENEWEDEEEDDQEDNDGGEAEESTESS
E. coli      VVMTADAVKQVEEMLA------------------------------
Chlamydomonas IIIEDEALAHVQSFYGAAAPASA-----------------------
             .......250.......260.......270.......280.....
```

FIGURE 3.—Alignment of ribosomal protein L4 from Chlamydomonas with the sequences from tobacco (GenBank T01739), Nostoc sp. PPCC 7120 (NP_488254), and *E. coli* (NP_417778) using Clustal X. The identities among the four sequences are indicated by an asterisk above the alignments. The identities among three of the four sequences are indicated by a period above the alignments. The similarities among the four sequences are indicated by a colon above the alignments. Amino acids G, P, S, and T are colored orange; amino acids H, K, and R are colored salmon; amino acids F, W, and Y are colored blue; amino acids D and E are colored purple; the amino acid P is colored yellow; and amino acids I, L, M, and V are colored green. The predicted chloroplast signal sequence is indicated by a black line below the alignments. The five sequenced *ery1* alleles have three changes. Three alleles (*ery1-3, ery1-5*, and *ery1-7*) have two different point mutations that change a glycine to an aspartic acid (shown in blue below the alignment). The *ery1-2* and *ery1-6* alleles have a splice site acceptor change as discussed in the text; the end of the affected exon is indicated by a blue +.

TUM and CHAMPNEY 1994). In resistant *E. coli*, the K_{63} is changed to a glutamic acid. Clearly, this is a region that is important for conferring resistance. In this $K_{63}E$ mutant in *E. coli*, erythromycin fails to bind to the ribosome (CHITTUM and CHAMPNEY 1995). The change from glycine to aspartic acid in *ery1-3* is consistent with the change in isoelectric point observed by DAVIDSON *et al.* (1974).

Isolation of ribosomes from *ery2* mutant strains suggested that the Ery2-4 protein had an alteration that was observable by one-dimensional gel electrophoresis with urea (METS and BOGORAD 1972, 1974), but the other mutant proteins had no alteration in their mobility. We are surprised that other alleles did not show alterations in these older studies as *ery2-2* and *ery2-3* alleles have amber codons that should produce truncated proteins. It is likely that all three mutant proteins are truncated.

We have used prediction programs to ask if RPL22 and RPL4 have signal sequence for import of the proteins into the chloroplast using ChloroP and TargetP (EMANUELSSON *et al.* 1999, 2000). Both programs predict signal sequences of 69 and 42 amino acids as indicated by the underlines in Figures 3 and 4. TargetP predicts that the signal sequences are mitochondrial rather than chloroplast import sequences. However, known Chlamydomonas chloroplast proteins, G3PD, RISP, and Photosystem I apoprotein A, also gave scores with TargetP that suggested the signal peptide was mitochondrial. This program is not optimized for signal sequences from Chlamydomonas, but remains useful. Alignment of RPL22 and RPL4 against bacterial proteins also suggests the presence of signal sequences. As shown in Figures 3 and 4, the first 60 and 42 amino acids showed little or no similarity to the protein from other organisms. This lack of similarity provides evi-
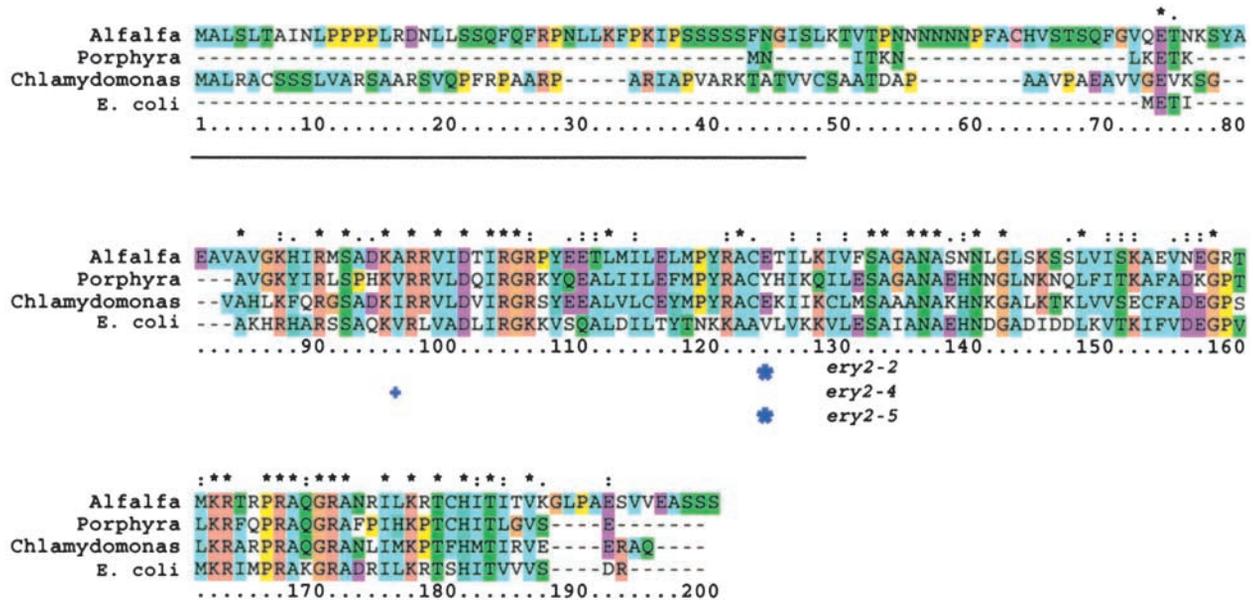
```
                                                                          *.
Alfalfa        MALSLTAINLPPPPLRDNLLSSQFQFRPNLLKFPKIPSSSSSFNGISLKTVTPNNNNNNPFACHVSTSQFGVQETNKSYA
Porphyra       ------------------------------------------MN---ITKN---------------LKETK---
Chlamydomonas  MALRACSSSLVARSAARSVQPFRPAARP-----ARIAPVARKTATVVCSAATDAP-------AAVPAEAVVGEVKSG--
E. coli        ---------------------------------------------------------------------METI----
               1......10........20........30........40........50........60........70........80

               *   :.  * *..*  *  *  *  ***:  .::*  :     :*.   :  :  :  ** ***.:* *   .* ::: .::*
Alfalfa        EAVAVGKHIRMSADKARRVIDTIRGRPYEETLMILELMPYRACETILKIVFSAGANASNNLGLSKSSLVISKAEVNEGRT
Porphyra       ---AVGKYIRLSPHKVRRVLDQIRGRKYQEALIILEFMPYRACYHIKQILESAGANAEHNNGLNKNQLFITKAFADKGYT
Chlamydomonas  --VAHLKFQRGSADKIRRVLDVIRGRSYVSQALDVLCEYMPYRACEKIIKCLMSAAANAKHNKGALKTLVVSECFADEGPS
E. coli        ---AKHRHARSSAQKVRLVADLIRGKKVSQALDILTYTNKKAAVLVKKVLESAIANAEHNDGADIDDLKVTKIFVDEGPV
               .........90.......100.......110.......120.......130.......140.......150.......160
                                                            *         ery2-2
                                                            +          ery2-4
                                                            *          ery2-5

               :**  ***:***  *  *  *  *:*:  *.      :
Alfalfa        MKRTRPRAQGRANRILKRTCHITITVKGLPAESVVEASSS
Porphyra       LKRFQPRAQGRAFPIHKPTCHITLGVS----E-------
Chlamydomonas  LKRARPRAQGRANLIMKPTFHMTIRVE----ERAQ----
E. coli        MKRIMPRAKGRADRILKRTSHITVVVS----DR------
               ......170.......180.......190.......200
```

FIGURE 4.—Alignment of ribosomal protein L22 (RPL22) from Chlamydomonas with the sequences from *M. sativa* (GenBank T09389), *P. purpurea* (NP_053919), and *E. coli* (NP_417774) using Clustal X. The identities among the four sequences are indicated by an asterisk above the alignments. The identities among three of the four sequences are indicated by a period above the alignments. The similarities among the four sequences are indicated by a colon above the alignments. Amino acids G, P, S, and T are colored orange; amino acids H, K, and R are colored salmon; amino acids F, W, and Y are colored blue; amino acids D and E are colored purple; the amino acid P is colored yellow; and amino acids I, L, M, and V are colored green. The predicted chloroplast signal sequence is indicated by a black line below the alignments. The *ery2-2* and *ery2-4* alleles have a stop codon that terminates the predicted proteins at the blue asterisk. The *ery2-5* allele has an altered splice site acceptor change as discussed in the text; the end of the affected exon is indicated by a blue +.

dence that this region corresponds to the signal sequence.

Erythromycin physically blocks the peptide exit tunnel (GABASHVILI *et al.* 2001). In *E. coli* mutant strains, L4 mutant ribosomes do not bind erythromycin and have a smaller tunnel size while L22 mutant ribosomes are able to bind erythromycin, but have a larger tunnel opening (GABASHVILI *et al.* 2001). On the basis of the crystal structure of the *H. marismortui* protein (HANSEN *et al.* 2002) and cryoelectron microscopy, RLP22 lines the peptide exit tunnel. The *ery2* alleles, which are predicted to result in truncated proteins, may create a peptide exit tunnel opening that is larger so that the erythromycin molecule cannot block the tunnel.

Erythromycin is one member of the macrolide family of antibiotics. It has a 14-membered lactone ring and one sugar group. Spiromycin and tylosin have 16-membered lactone rings and two sugar groups attached. X-ray crystallography of ribosomes from *H. marismortui* in the presence of these antibiotics shows that these compounds form covalent bonds with the 23S rRNA. The forosamine sugar moiety of spiromycin contacts protein L4 and the mycinose sugar moiety of tylosin lies along the peptide exit tunnel and contacts protein L22 (HANSEN *et al.* 2002). The *ery1* mutations in RPL4 confer resistance to both of the compounds, but the *ery2* mutations in RPL22 confer resistance only to tylosin. It is

possible that the interaction of the mycinose sugar moiety of tylosin positions it so that the absence of the carboxy terminus of RPL22 is not sufficient to open the peptide exit tunnel. We modeled the sequence of RPL22 from Chlamydomonas onto the structure of RPL22 from *H. marismortui* using SwissModel (GUEX *et al.* 1999). There are three major differences between the known structure and the modeled structure for Chlamydomonas (Figure 5). First, the divergent amino terminus of the protein cannot be predicted from the crystal structure. Second, one of the helices in *H. marismortui* is missing from the modeled structure. Third, the angle of the extended loop is different (Figure 5A). To show the extent of the truncation predicted in the *ery2-2* or *ery2-4* alleles, the wild-type model (in green) is superimposed on the truncated model (in black). The loop, which is thought to line the peptide exit tunnel, is missing. At present, we have no biochemical data to support the idea that the truncated protein is present.

The region of RPL4 that contains the glycine-to-aspartic acid mutations at 102 and 112 was not modeled onto the *H. marismortui* L4 protein as this region was unordered and is not in the crystal structure. It is reasonable to suspect that this region forms a hydrophobic face and that the addition of aspartic acid to this face would disrupt it and possibly block erythromycin binding.
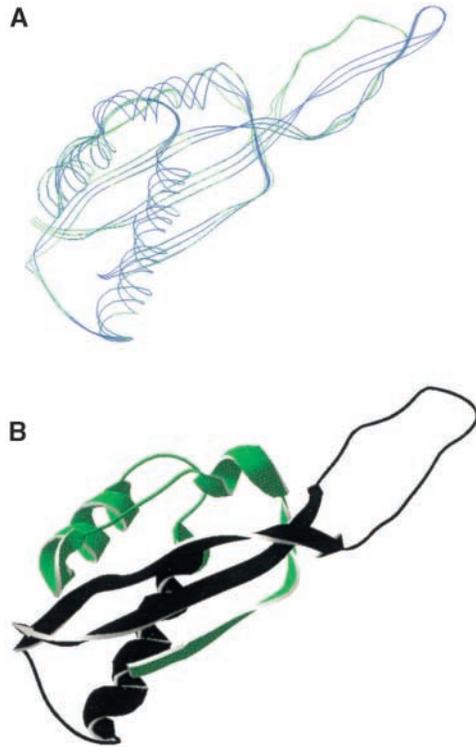
Figure 5.—Predicted structure of L22 from Chlamydomonas using SwissModel. (A) The confirmation of L22 from *H. marismortui* (in blue) and the predicted structure of L22 from Chlamydomonas (in green) are shown. Several differences are seen between the two structures. The first 60 amino acids of Chlamydomonas L22 were not used in modeling. One of the helices in *H. marismortui* is significantly different in the modeled structure as seen at about 11 o'clock. In addition, the angle of the extended loop is different. (B) The predicted protein from the *ery2-2* allele (green) is compared to the full-length L22 protein (black) from Chlamydomonas to show the extent of the truncation.

Strains with the *ery2* mutation may serve as an excellent recipient for transformation as one could select both positively and negatively for the different alleles. The mutant strain is unable to grow at 15°, which would allow for selection of the wild-type *ERY2* DNA while resistance to erythromycin at 25° is dominant to the wild-type allele (Hanson and Bogorad 1977).

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