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Research Article

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Inhibition of Cytoplasmic and Organellar Protein Synthesis in *Toxoplasma gondii*

Implications for the Target of Macrolide Antibiotics

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Abstract

We investigated potential targets for the activity of protein synthesis inhibitors against the protozoan parasite *Toxoplasma gondii*. Although nanomolar concentrations of azithromycin and clindamycin prevent replication of *T. gondii* in both cell culture and in vivo assays, no inhibition of protein labeling was observed in either extracellular or intracellular parasites treated with up to 100 μM drug for up to 24 h. Quantitative analysis of > 300 individual spots on two-dimensional gels revealed no proteins selectively depleted by 100 μM azithromycin. In contrast, cycloheximide inhibited protein synthesis in a dose-dependent manner. Nucleotide sequence analysis of the peptidyl transferase region from genes encoding the large subunit of the parasite's ribosomal RNA predict that the cytoplasmic ribosomes of *T. gondii*, like other eukaryotic ribosomes, should be resistant to macrolide antibiotics.

Combining cycloheximide treatment with two-dimensional gel analysis revealed a small subset of parasite proteins likely to be synthesized on mitochondrial ribosomes. Synthesis of these proteins was inhibited by 100 μM tetracycline, but not by 100 μM azithromycin or clindamycin. Ribosomal DNA sequences believed to be derived from the *T. gondii* mitochondrial genome predict macrolide/lincosamide resistance. PCR amplification of total *T. gondii* DNA identified an additional class of prokaryotic-type ribosomal genes, similar to the plastid-like ribosomal genes of the *Plasmodium falciparum*. Ribosomes encoded by these genes are predicted to be sensitive to the lincosamide/macrolide class of antibiotics, and may serve as the functional target for azithromycin, clindamycin, and other protein synthesis inhibitors in *Toxoplasma* and related parasites. (*J. Clin. Invest.* 1995. 95:367–376) Key words: *Toxoplasma gondii* • macrolides • protein synthesis • plastid • ribosomes

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Introduction

Several groups of antimicrobial agents which inhibit protein synthesis on prokaryotic ribosomes exhibit potent activity against *Toxoplasma gondii* both in vitro and in vivo (reviewed in [1]). These agents include drugs of the macrolide, azalide, and lincosamide classes, which bind to 50S ribosomal subunits, and drugs of the tetracycline class, which bind to both large and small ribosomal subunits, but have greatest avidity for the 30S subunit. Some such compounds have proved effective in therapy of clinical toxoplasmosis, but their target site(s) and mechanism(s) of action against *T. gondii* remain unclear.

Extremely low concentrations of clindamycin or azithromycin (10 and 30 ng/ml, respectively) are sufficient to inhibit parasite growth in extended tissue culture assays (2, 3), and it has been suggested that the activity of azithromycin against *Toxoplasma* results from the inhibition of total protein synthesis (4). The observation that > 100-fold higher drug concentrations are needed to even partially inhibit total protein synthesis in extracellular parasites (3) has been taken to imply that parasites growing inside the parasitophorous vacuole must concentrate these compounds extensively within the parasite cytoplasm (4). We have recently demonstrated that azithromycin taken up by *T. gondii*–infected fibroblasts is indeed localized, but that the drug is concentrated within lysosomes of the host cell and in acidified organelles of the parasite (5). Azithromycin levels in the parasite cytosol are elevated only two to threefold over extracellular levels. At typical doses, the drug would therefore reach maximal concentrations of 2–3 μg/ml, far short of the levels needed to inhibit protein synthesis on cytoplasmic ribosomes.

We have therefore reexamined possible target sites for protein synthesis inhibitors with activity against *T. gondii*. By determining the pattern of protein synthesis in extracellular and intracellular parasites, and the primary nucleotide sequence of ribosomal RNA genes from nuclear and putative organellar ribosomes, we have developed a unifying hypothesis for the activity of protein synthesis inhibitors against *T. gondii*. None of the compounds under study is likely to act against cytoplasmic ribosomes of the parasite, which are typically eukaryotic in primary structure (6, 7).[1] Rather, these drugs probably act against prokaryotic type ribosomes. In addition to mitochondrial ribosomes, evidence has been obtained for a novel class of ribosomal genes related to the plastid-like sequences recently identified in *Plasmodium* species (8, 9). These experiments have considerable relevance for antimicrobial therapy against

1. Luft, B. J., et al., manuscript in preparation.

all Apicomplexan parasites (10), since they suggest a novel antimicrobial target probably common to all members this group.

Methods

Buffers and reagents. HBSS was supplemented with 3.5 mg/ml BSA and 5–10 mM Hepes buffer. AISS buffer (11) contained 120 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM Hepes (unless otherwise noted). AISS+ buffer consisted of AISS supplemented with 1 × MEM essential and nonessential amino acids (without methionine), 1 × MEM vitamins, 1 mM Na-pyruvate, 1 mM ATP, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. [^{35}S]methionine (specific activity > 1000 Ci/mmol) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Azithromycin was a gift from Pfizer Central Research (Groton, CT). Cycloheximide, erythromycin, chloramphenicol, tetracycline, clindamycin, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Parasites. A clonal derivative of the virulent RH strain of *Toxoplasma gondii* was used throughout these experiments (originally provided by Dr. Melvin Lundy, National Institute of Health (NIH). An azithromycin-resistant mutant derived from the RH strain (3) was kindly provided by Dr. Elmer Pfefferhorn (Dartmouth College). Parasite tachyzoites were harvested from mouse peritoneal fluid or from human fibroblast monolayers as previously described (12). Parasites harvested from mice were passed through a 27-gauge needle and a 3-μM polycarbonate filter (13) to release intracellular *T. gondii* and remove contaminating mouse peritoneal cells.

Susceptibility studies. The susceptibility of parasite tachyzoites to azithromycin was measured by inoculating parasites onto monolayers of human foreskin fibroblasts grown on glass coverslips, at a parasite:cell ratio of ~ 1:10. After 2 h, azithromycin was added at various concentrations up to 3 μg/ml, and incubation was continued at 37°C in 5% CO_2 for 4 d. Coverslips were washed in HBSS, fixed with 3% paraformaldehyde, and the number of intracellular parasites scored by phase contrast microscopy. Quantitation was performed by counting the total number of parasites (intracellular plus extracellular) present in a given culture (typically 2.5×10^6 for untreated controls). The IC_{50} for azithromycin treatment of wild-type parasites was < 30 ng/ml, while the 50% inhibiting drug concentration (IC_{50}) against an azithromycin-resistant mutant (3) was > 300 ng/ml. In contrast, the IC_{50} for tetracycline was > 2 μg/ml, approximately two orders of magnitude higher than observed for azithromycin (or clindamycin).

[^{35}S]methionine incorporation into extracellular tachyzoites. Inhibition of protein synthesis was measured in extracellular tachyzoites using ionic conditions optimized as described in the text. The effect of monovalent cations on tachyzoite motility (14), infectivity (15), and protein synthesis (16) has been previously noted. 10^7 extracellular parasites were added to 1-ml aliquots of a series of modified pH 7.2 AISS buffers (containing 0.1% BSA), in which the KCl/NaCl ratio was varied, but the sum of the NaCl and KCl concentrations kept constant at 150 mM. After a 30-min preincubation at 37°C, 10 μCi of [^{35}S]-methionine was added to each aliquot and incubation continued for 60 min. 100-μl aliquots were removed and organisms lysed in 0.05% deoxycholate. Nucleic acids were hydrolyzed for 15 min at 37°C by the addition of 25 μl of 10 N NaOH, and proteins precipitated with 0.5 ml of 25% TCA for 30 min on ice. The precipitate was isolated by filtration, washed with 10 ml of ice-cold 5% TCA followed by acetone, and radioactivity quantitated by liquid scintillation counting.

To confirm that parasite protein synthesis was being measured (rather than protein synthesis from host cell contaminants), metabolically labeled proteins from parasites and from uninfected human foreskin fibroblast cells were compared by two-dimensional gel electrophoresis, before and after immunoprecipitation with antiserum to whole *T. gondii*, as previously described (17). Greater than 98% of the metabolically labeled spots in extracellular parasites were of parasite origin.

The effects of the protein synthesis inhibitors (cycloheximide at 0.01–100 μM [2.8 ng/ml–28.1 μg/ml], erythromycin at 100 μM [73.4 μg/ml], chloramphenicol at 300 μM [96.9 μg/ml], tetracycline at 0.1–100 μM [48 ng/ml–48 μg/ml], and azithromycin at 0.1–100 μM [78.5 ng/ml–78.5 μg/ml]) were tested using the [^{35}S]methionine incorporation assay described above, after 30 min preincubation in drug. As a positive control for antibiotic efficacy, [^{35}S]methionine incorporation into *Staphylococcus aureus* (25923; American Type Culture Collection obtained from P. Marone, Yale University) was inhibited by 97% in 1-μM azithromycin, and by 92% in 10-μM tetracycline; 100-μM cycloheximide had no effect against *S. aureus*.

[^{35}S]methionine incorporation in infected and uninfected human fibroblasts. Human fibroblasts were grown to confluence in six-well dishes and infected with *T. gondii* at a parasite:cell ratio of 5:1 in αMEM containing Na-pyruvate, glutamine, and 10% fetal calf serum. Under these conditions, > 80% of cells were infected, typically with a single vacuole containing eight parasites 24 h after inoculation. Infected monolayers were washed once in PBS, and twice in methionine-free DMEM medium (GIBCO BRL, Gaithersburg, MD) containing 1-mM pyruvate, 2-mM glutamine and 3% dialyzed fetal calf serum. Antibiotics were added to the desired concentration in 1 ml of methionine-free medium (supplemented as above), and cells were incubated at 37°C in a 5% CO_2 environment. After 30 min, 10 μCi of [^{35}S]methionine was added per well, and incubation continued for an additional 2 h with periodic shaking. Cells were washed twice in Ca/Mg-free PBS, scraped into PBS, pelleted by centrifugation, and solubilized in sample buffer for analysis by two-dimensional gel electrophoresis. Uninfected cells were always labeled and processed in parallel.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis (18) was performed using 1% (vol/vol) ampholyte 3/10 and 4% (vol/vol) ampholyte 5/8 in the sample buffer and first dimension. Samples were solubilized in 50 μl isoelectric focusing sample buffer per 10^7 extracellular parasites or 10^6 infected human fibroblast cells. Samples were separated in the first dimension by isoelectric focusing (16 h at 400 V, 1 h at 800 V) in gels of 0.15 × 14 cm cast in glass tubes. Second dimension SDS-PAGE was performed with the PROTEAN II Xi Multi-Cell system (Bio-Rad Laboratories; Hercules, CA), using 7.5, 10, or 12.5% SDS-polyacrylamide gels. For calibration, standards (Bio-Rad Laboratories) were run on a parallel gel and detected by silver staining. Completed gels were incubated 15 min in 30% methanol containing 0.125 M sodium salicylate, dried on 3MM paper (Whatman Inc., Clifton, NJ) and exposed to XAR5 film (Eastman Kodak Co., Rochester, NY) at −70°C. Autoradiograms were scanned using the BioImage system Visage 2000 (BioImage, Ann Arbor, MI) to identify spots automatically, with manual interaction to confirm questionable spots or gel artifacts. Different exposures were used to ensure that spots over a broad range of intensities were analyzed.

Nucleotide sequence analysis of peptidyl transferase region of T. gondii *ribosomes.* Sequences encompassing the peptidyl transferase region (domain V) of the *T. gondii* RH strain large subunit (LSU)[2] ribosomal RNA (rRNA) genes were amplified by polymerase chain reaction using consensus primers for various eukaryotic and prokaryotic ribosomes. For cytoplasmic ribosomes, primers for a 2.0-kb internal fragment of the LSU extending into the peptidyl transferase domain were based on the sequence of the corresponding region of the dinoflagellate *Prorocentrum micans.* (GenBank sequence X16108 [19]), as the *P. micans* small subunit rRNA sequence had previously been shown to be relatively similar to *T. gondii* (6, 7). Primers 5′-GGTAAAGCGAATGATTAGAGG-3′ and 5′-TCTCACGACGGTCTAAACCCA-3′ correspond to bases 858–877 and 2600–2580, respectively in the *Escherichia coli* LSU sequence based on alignments in the Ribosomal Database Project (20); these primers correspond to bases 1051–1071 and 3005–2985 in the GenBank *P. micans* listing (19).

As the *T. gondii* rRNA genes are organized in a tandem head-to-tail dimer of alternating small and large subunit genes (with intervening

2. *Abbreviations used in this paper:* LSU, ribosomal large subunit; nt, nucleotide; PI, isoelectric point.

5S and 5.8S genes; GenBank X75429), the remainder of the peptidyl transferase sequence was amplified as a 2.7-kb fragment using a sense primer internal to the peptidyl transferase region (5′-CGGATTGTT-CACCCGCCA-3′; *E. coli* LSU nucleotide (nt) 2547–2564; *P. micans* LSU nt 2952–2969) and an antisense primer derived from the 5′ end of the small subunit ribosomal sequence (5′-AGACAAGCATATGAC-TAC-3′; *E. coli* small subunit nt 42–25; *P. micans* small subunit nt 36-19).

Amplification conditions for the above PCR reactions were as follows: 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and elongation at 72°C for 3 min in a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). Amplified fragments were purified using a DNA purification system (Magic PCR; Corp.; Promege, Madison, WI), cloned into the PCRII vector (Invitrogen San Diego, CA) according to the manufacturers instructions, and sequenced using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH).

The peptidyl transferase region of nonmitochondrial prokaryote-like ribosomal genes was also identified using a two-step PCR strategy. Based on examination of sequences flanking the transpeptidation domain in the Ribosomal Database (20), we synthesized a sense-orientation primer predicted to hybridize ~ 100 nt upstream of the transpeptidation domain (primer L2: 5′-GCGAAATTCCTTGTCGGGTAAGTT-CC-3′; corresponding to nt 1097–1132 in the *E. coli* sequence), and an antisense primer (L3: 5′-ATCCCTAGAGTAACTTTATCCGT-3′; nt 2449–2426 in the *E. coli* sequence) predicted to hybridize in the middle of the transpeptidation domain. Primer L2 is expected to recognize all prokaryotic LSU rRNA genes, while primer L3 should recognize plastid-like, mitochondrial, and eubacterial ribosomal genes, but not archaebacterial genes.

To clone the 3′ end of the transpeptidation domain, a new sense-orientation primer specific for the plastid-like genome of *Toxoplasma* was designed based on sequence information obtained from the L2–L3 reactions above (L11: 5′-TAGTGATCCGACAACTCTAAG-TGGT-3′; nt 2387–2411 in the *E. coli* sequence). This primer was used in conjunction with a universal antisense primer expected to hybridize downstream of the transpeptidation domain in all ribosomes, both eukaryotic and prokaryotic (L4: 5′-TTTYYGWTCCTCTCGTAC-3′ [Y=C/T; W=A/G]; nt 2672–2655 in the *E. coli* sequence).

PCR reactions were carried out at low stringency in 200–μl thin-walled reaction tubes (Perkin-Elmer Cetus Instruments), using 100 ng total parasite DNA (strain RH) and 100 pmol of each primer (L2 + L3 or L2 + L4). *Taq* polymerase (Promega Corp.) was added at 85°C, and the reaction cycled 35 times through the following program: 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C, in a minicycler (M Research Inc., Watertown, MA). Reaction products were cloned directly in vector pCRII (Invitrogen), and sequenced from the flanking T7 and Sp6 polylinker primers using Sequenase 2.0 (United States Biochemical Corp.). To minimize the possibility of errors introduced by the low fidelity of *Taq* polymerase, all PCR reactions were carried out using high concentrations of genomic parasite DNA (typically 100 ng), and at least two independently amplified clones were sequenced for each primer pair. No sequence discrepancies were noted in the transpeptidation domain shown.

Results

Cytoplasmic protein synthesis is not sensitive to azithromycin

Macrolide antibiotics have previously been reported to inhibit protein synthesis in extracellular tachyzoites only at concentrations exceeding 20 μg/ml. These levels are nearly two orders of magnitude greater than required for killing intracellular parasites (3, 4), and an order of magnitude higher than achieved in the parasite cytosol with usual doses (5). Although these results suggest that cytoplasmic ribosomes are not inhibited under physiological conditions, an alternative is to postulate that protein synthesis and sensitivity to inhibitors differs dramatically between the extracellular and intracellular forms of *T. gondii* tachyzoites.

Table I. Effect of Protein Synthesis Inhibitors on [^{35}S]Methionine Incorporation by T. gondii

Drug	Concentration	[^{35}S]Methionine incorporation (percent control)*	
		60 min‡	24 hr§
	μM		
Cycloheximide	100	0.5‖	ND
	10	1.9±2.1	3.1±1.6
	1	12±1.4	ND
	0.10	39‖	ND
	0.01	79‖	ND
Azithromycin	100	100±14	96±9
Clindamycin	100	94±7	86±13
Erythromycin	100	104±3	ND
Chloramphenicol	300	101±1	94±4
Tetracycline	10	ND	87±11
	100	91±4	56±3

* Values represent means±SD for two to five experiments carried out in triplicate. ‡ Experiments carried out in AISS medium. § Experiments carried out in AISS medium supplemented with amino acids, pyruvate, vitamins, and ATP, to preserve parasite viability during long-term incubation. ‖ Single determination. ND, not done.

Optimization of protein synthesis in extracellular parasites. To determine whether the drug sensitivity of cytoplasmic ribosomes might be altered under the ionic environment prevailing within infected host cells, we modified parasite incubation conditions to more closely mimic the intracellular milieu. Replicating *T. gondii* tachyzoites reside in a vacuole inside cells which is permeable to small molecules and therefore likely to have an ionic composition closely resembling that of cell cytosol (21). We tested the effect of buffer ion composition on total protein synthesis in extracellular tachyzoites, comparing extracellular and intracellular type buffers. Protein synthesis was highly sensitive to extracellular potassium concentration, incorporating 2.5-fold more [^{35}S]methionine in buffers containing > 75 mM K^+ than in buffers containing < 25 mM K^+. These data indicate that a cytosol-like buffer is optimal for protein synthesis in *T. gondii.* Aside from the total level of metabolic labeling observed, however, the profile of proteins labeled during growth in αMEM or AISS buffer was indistinguishable by quantitative two-dimensional gel electrophoresis (not shown).

The effect of various antibiotics on [^{35}S]methionine incorporation into *T. gondii* protein was measured during incubation of extracellular tachyzoites (Table I). Submicromolar concentrations of cycloheximide, a classic inhibitor of eukaryotic protein synthesis, (22) effectively blocked methionine incorporation by extracellular *T. gondii* (Table I). In contrast, chloramphenicol, an inhibitor of protein synthesis on prokaryotic ribosomes, had no effect at 300 μM. Neither was any significant effect seen using azithromycin, clindamycin, or erythromycin at a concentration up to 100 μM. These concentrations are far in excess of achievable plasma levels and would not be expected in the cytosol of the intracellular tachyzoite (5). Partial inhibi-

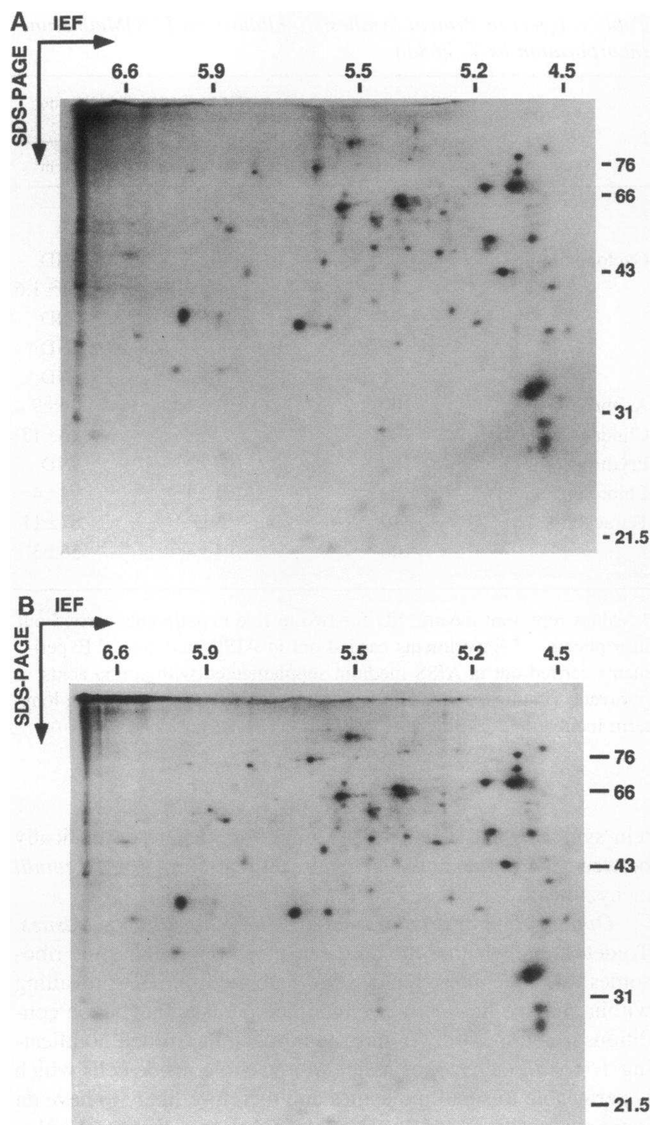


Figure 1. Two-dimensional gel profile of ^{35}S-labeled proteins synthesized in extracellular RH strain tachyzoites in the absence or presence of azithromycin. Extracellular parasites were incubated with buffer alone (*A*), or with 10 μM (*B*) azithromycin for 2 h at 37°C. The profile of [^{35}S]methionine labeled proteins on two-dimensional 12.5% polyacrylamide gels was determined as described in Methods. Gels were exposed to x-ray film overnight at −80°C. No reproducible differences from controls were detected in the presence of azithromycin.

tion was observed after long periods of incubation in 10–100 μM tetracycline; the basis for the delayed effect remains unclear.

Two-dimensional gel analysis of protein synthesis. Although azithromycin (and other inhibitors of prokaryotic ribosomes) failed to suppress total methionine incorporation, it was still possible that the spectrum of proteins synthesized was altered by these inhibitors. We therefore examined two-dimensional gel profiles of metabolically labeled protein in parasites treated with increasing concentrations of azithromycin and compared these results with the profile from untreated parasites, as shown in Fig. 1. On a 12.5% gel, over 300 spots varying in isoelectric point between 4.3 and 6.6 and in M_r between 15 and 100 kD were identified in untreated extracellular parasites (*A*). After treatment with 10 μM azithromycin (*B*), no reproducible differences in the spectrum or intensity of spots were detected by careful visual inspection or by automated analysis. Neither were any differences detected using 1 or 100 μM azithromycin (not shown).

Protein synthesis in intracellular parasites. As noted above, macrolide antibiotics had no detectable effect on parasites incubated in either normal culture medium or under intracellular buffer conditions. Even in AISS buffer, however, parasites fail to replicate extracellularly, and it therefore remains a formal possibility that critical parasite proteins—synthesized on cytoplasmic ribosomes only during intracellular replication—are sensitive to macrolide treatment. To investigate this scenario, host cell cultures were infected with parasites, treated with azithromycin, and examined by quantitative two-dimensional gel electrophoresis as shown in Fig. 2. Although the presence of large numbers of host cell proteins greatly complicates analysis, > 140 parasite-specific proteins were readily identified. Based on quantitative two-dimensional gel digitization, none of the proteins visualized, including those of parasite origin, was sensitive to azithromycin treatment at concentrations up to 100 μM, as shown in Fig. 2 *C*.

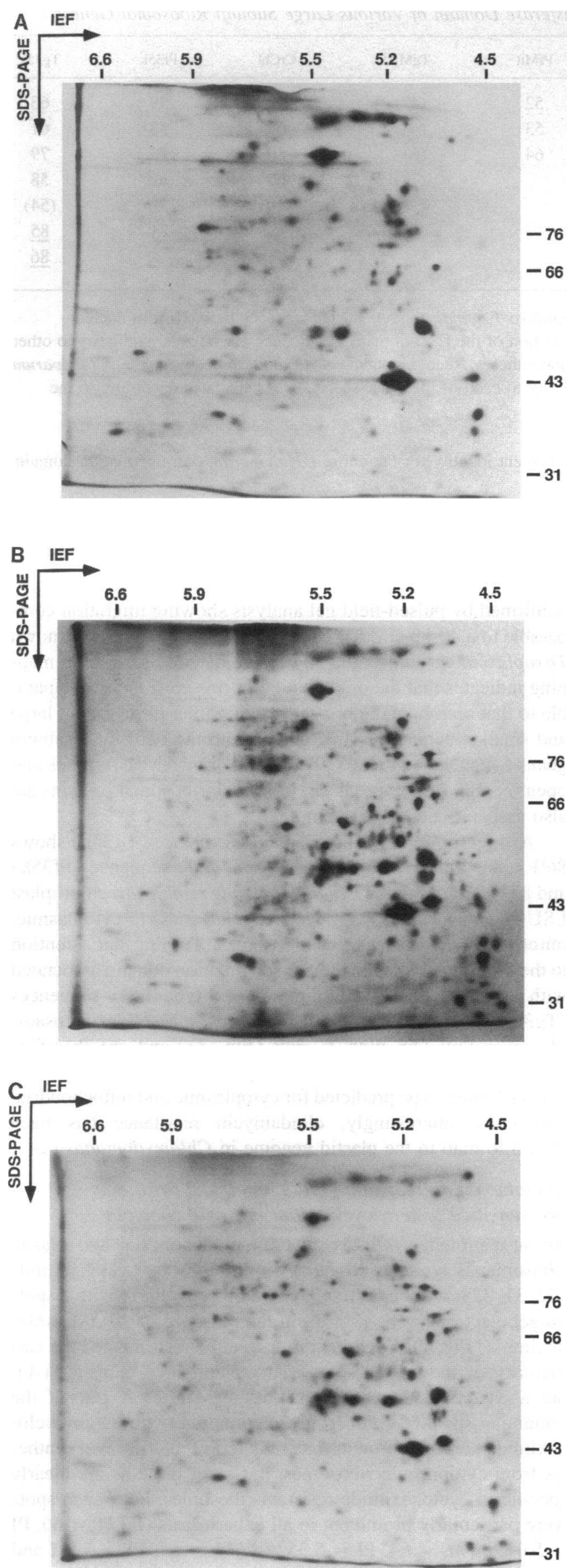
Primary sequence of the peptidyl transferase region of T. gondii cytoplasmic ribosomes. Unlike some protein synthesis inhibitors (e.g., tetracyclines), which interact with ribosomal proteins in a manner independent of the primary rRNA sequence, macrolide resistance is often associated with specific point mutations in the peptidyl transferase region of the ribosomal large subunit (23, 24).

PCR primers based on LSU sequence from the dinoflagellate *P. micans* were used to amplify an internal 2-kb fragment from *T. gondii* genomic DNA, as described in Methods. (The complete sequence of the *T. gondii* small and large subunit rRNA genes have been deposited with EMBL [×75429]; detailed analysis and predicted secondary structure will be published elsewhere. As expected, the *Toxoplasma* rRNA sequence is clearly eukaryotic in nature. Comparing 218 nt from the transpeptidation domain, which can be unambiguously aligned, the human and *Toxoplasma* cytoplasmic ribosomes share 94% sequence identity, while *Toxoplasma* and *E. coli* exhibit only 64% identity (Table II). Nucleotide sequences covering the known macrolide/lincosamide sensitivity loci within the peptidyl transferase domain are shown in Fig. 3. At least three positions in the *T. gondii* ribosomal gene sequence, A_{2057}, A_{2058}, and U_{2611}, involve differences known to confer lincosamide or macrolide resistance to prokaryotic ribosomes (23, 24).

Taken as a whole, the metabolic data on protein labeling in both intracellular and extracellular parasites, combined with ribosomal sequence analysis, suggest that macrolides and lincosamides do not inhibit protein synthesis on cytoplasmic ribosomes of *Toxoplasma.* It is possible that these drugs are toxic to *T. gondii* for reasons independent of protein synthesis, but this seems unlikely (see Discussion). We must, therefore, consider alternative ribosomal targets.

T. gondii contains at least two noneukaryotic ribosomal genes

Mitochondrial ribosomal sequences. T. gondii parasites contain a single prominent mitochondrion, but the complete mitochondrial genome has not been identified, in part because the parasite's nuclear DNA contains large numbers of what appear to be



retro-transposed mitochondrial sequences (25). Nevertheless, from these fragments of mitchondria-like sequence in the nuclear genome, it is possible to piece together parts of the predicted mitochondrial peptidyl transferase domain by homology with other mitochondrial LSU sequences. Such pseudogenes must be considered with caution, as they may have acquired mutations unrelated to the actual *T. gondii* mitochondrial genome. The sequence TgMt? in Fig. 3 derives from two independent nuclear retro-transposons which are identical across the region shown, however, raising confidence in their fidelity to the true mitochondrial sequence.

The predicted partial *T. gondii* mitochondrial sequence also exhibits strong similarity to the proven mitochondrial genome of the related Apicomplexan parasite *P. falciparum* (designated PfMit; (26): 78% identity across the major macrolide/lincosamide sensitivity domain (Table II). Compared with the *E. coli* sequence and known drug-resistance mutations in several organelles and prokaryotes (23, 24), the presence of nucleotides A_{2032} and U_{2058} in the mitochondrial sequence of both *Toxoplasma* and *Plasmodium* suggests that these ribosomes are resistant to macrolides. It is interesting to note that while some mitochondrial ribosomes are predicted to be macrolide sensitive (including the ciliates such as *Paramecium tetraurelia* (27), mitochondrial ribosomes of both animals, including humans, and plants are predicted to be macrolide resistant. The predicted *Toxoplasma* sequence also differs from *E. coli* at U_{2059}, but the significance of this alteration is unclear, as it has not been observed in other mutant or wild-type sequences where macrolide susceptibility is known. We have not been able to determine the nucleotide base at position 2611, perhaps because this region (indicated by question marks in Fig. 3) has not been included in any of the mitochondrial retrotransposons sequenced to date, or perhaps because the extreme fragmentation of mitochondrial ribosomal genes in the Apicomplexa prevent identification through sequence alignment. Because this nucleotide normally basepairs with nucleotide 2057 (20, 28), however, we would predict a C at this locus.

Plastid-like ribosomal genes in Toxoplasma. Studies on the malaria parasite *P. falciparum* have recently revealed a novel extrachromosomal element most closely related to the genome of plastids (8, 9). In retrospect, it seems clear that this genome represents the homologue of a molecule previously identified in electron microscopic spreads of *Toxoplasma* DNA (29). To determine the nucleotide sequence and predicted drug sensitivity of this putative organellar genome, we designed a variety of prokaryote-specific PCR primers targeted to the ribosomal genes. Low stringency amplification reactions (hybridization at 42°C) were carried out for the L2–L3 and L11–L4 primer pairs (see Methods). Products of the expected sizes were obtained from both reactions, but not from single primer reactions or a host cell (human) nuclear DNA control.

Analysis of these PCR products identified overlapping sequences homologous to the malaria plastid-like ribosomal genes

Figure 2. Two-dimensional gel profile of proteins from human fibroblasts infected with *T. gondii* strain RH in the absence or presence of antibiotics. (*A*) uninfected fibroblasts. (*B*) infected fibroblasts. (*C*) infected fibroblasts treated with 100 μM azithromycin. To optimize the visualization of the host and parasite proteins, all samples were electrophoresed on 7.5% gels in the second dimension, in contrast to the 12.5% gels used in Figs. 1 and 4. Gels were developed for 2 d at −80°C.

*Table II. Percent Nucleotide Sequence Identity across the Peptidyl Transferase Domain of Various Large Subunit Ribosomal Genes**

Species	Location	TgCyt	*E. coli*	PfMit	TgMt?[‡]	CrChl	Pf35k	Tg35k
H. sapiens	Cytoplasmic	94[§]	64	52	(47)	63	59	63
T. gondii	Cytoplasmic		64	53	(48)	63	58	61
E. coli	Bacterial			64	(61)	80	75	79
P. falciparum	Mitochondrial				(78)[‖]	60	62	58
T. gondii	Mitochondrial[‡]					(50)	(58)	(54)
C. reinhardtii	Chloroplast						81	85
P. falciparum	35 kb/Plastid?							86

* See text and Fig. 3 for sources and details on nomenclature. [‡] Based on putative *T. gondii* mitochondrial sequence derived from nuclear retrotransposons ([25] and Roos, D.S., unpublished observations). As only a portion of the LSU domain V sequence is available, similarity to other sequences over the entire domain must be estimated and is therefore shown in parentheses. Recognizing the close similarity between the *P. falciparum* mitochondrial gene and the putative *T. gondii* mitochondrial sequence, similarity to each of the other species was calculated according to the following formula:

$$\frac{\text{percent identity for the available } T.\ gondii \text{ mitochondrial sequence}}{\text{percent identity for } P.\ falciparum \text{ mitochondrial sequence in same region}} \times \text{percent identity for the entire } P.\ falciparum \text{ transpeptidation domain}$$

[§] Underlined numbers indicate that these sequences show greater similarity to each other than either sequence exhibits to *E. coli.* [‖]Percent identity based on 38 nucleotides of putative *T. gondii* mitochondrial sequence spanning the major macrolide/lincosamide sensitivity domain.

and spanning the transpeptidation domain. Their hybridization pattern on Southern blots of total *Toxoplasma* genomic DNA is consistent with a 30–35-kb circle (Donald, R. G. K., and D. S. Roos, manuscript in preparation). This interpretation is confirmed by pulsed-field gel analysis showing migration comparable to a 40-kb circular cosmid, but distinct from any known *Toxoplasma* chromosome (30). More detailed restriction mapping indicates that the organization of this episome is comparable to that seen in malaria: a tail-to-tail dimer of divergent large and small subunit ribosomal genes, with the two small subunit genes facing each other (8, 9). Several transfer RNA genes and open reading frames predicted to encode ribosomal proteins are also associated with this complex.

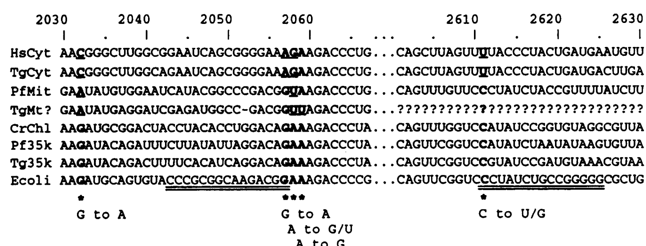
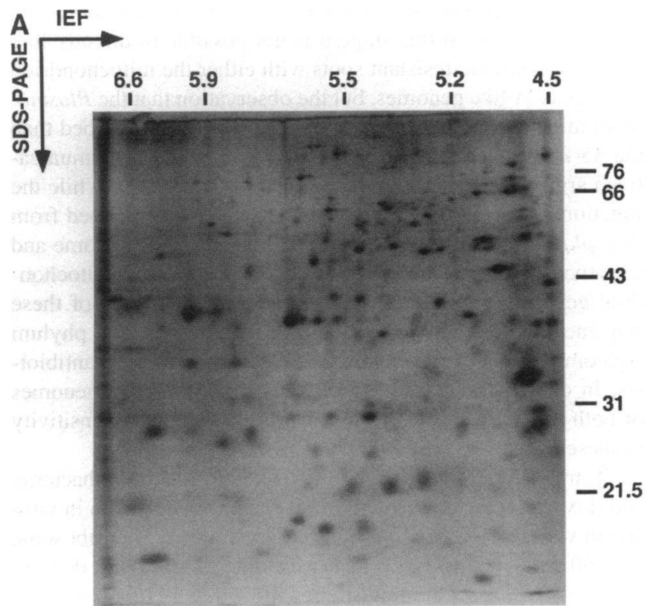


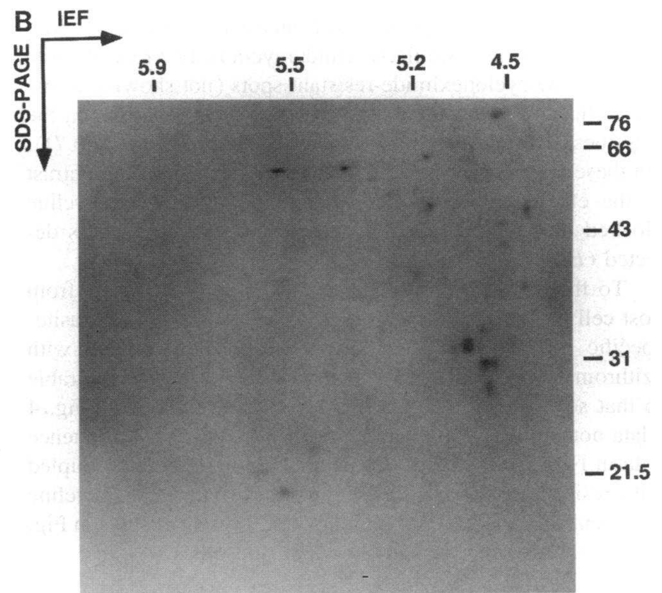
Figure 3. Nucleotide sequence of lincosamide sensitivity loci from *T. gondii* ribosomal genes, compared with other nuclear and organellar ribosomes. *Toxoplasma* sequences were obtained as described in the text; other sequences are available through GenBank: HsCyt, Homo sapiens cytoplasmic ribosome (GenBank M27830), TgCyt, *T. gondii* cytoplasmic ribosome (this report; EMBL X75429); PfMit, *P. falciparum* mitochondrial ribosome (M21313); TgMt?, *T. gondii* mitochondrial ribosome (putative, see text; X60242; question marks indicate sequence not available); CrChl, *Chlamydomonas reinhardtii* chloroplast ribosome (X15727, X16686); Pf35k, *P. falciparum* 35-kb plastid-like ribosome (X61660); Tg35k, *T. gondii* plastid-like genome (this report; GenBank U18086); *E. coli, Escherichia coli* ribosome (V00331). Sequences surrounding the lincosamide sensitivity region in the peptidyl transferase domain (LSU, Region V) were identified by comparison with alignments obtained from the Ribosomal Database Project, and numbered according to the *E. coli* sequence. The double-underlined region indicates basepairing of the transpeptidation domain stem (C_{2043}–G_{2057} basepairs with G_{2625}–C_{2611} in the folded ribosome [28]). Loci known to be associated with drug resistance (23, 24) are indicated with asterisks, and the particular mutations thought to confer resistance are noted beneath. Underlined nucleotides in the various sequences shown differ from the *E. coli* (drug-sensitive) sequence at the relevant positions. Note that both the cytoplasmic and putative mitochondrial ribosomal sequences from *T. gondii* are predicted to be drug resistant, while the plastid-like ribosomal genes appear likely to be sensitive to clindamycin and macrolide antibiotics.

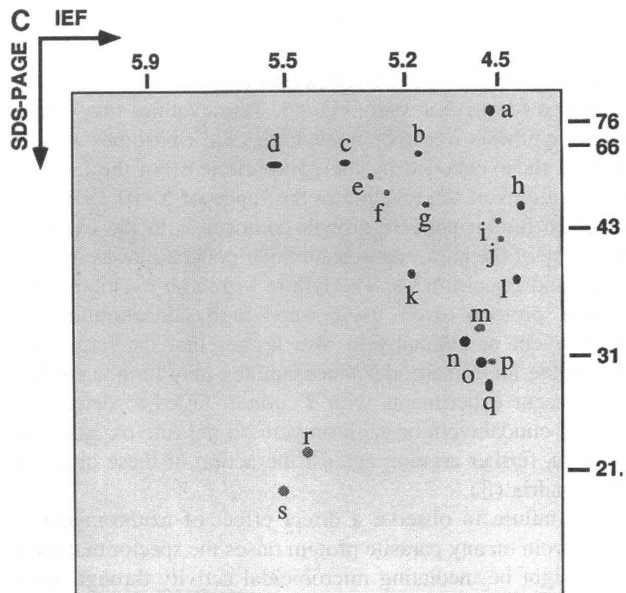
As seen in Table II, the *T. gondii* sequence (Tg35k) shows 86% nucleotide identity to the *Plasmodium* sequence (Pf35k) and 85% identity to the *Chlamydomonas reinhardtii* chloroplast LSU (CrChl), with lower levels of relatedness to cytoplasmic, mitochondrial, and bacterial ribosomes. Turning our attention to the specific loci within the transpeptidation domain associated with drug resistance, the three plastid-type LSU sequences (Tg35k, Pf35k, and CrChl) share all five macrolide/lincosamide sensitivity loci with *E. coli* (Fig. 3), and are therefore predicted to be sensitive to these drugs, in marked contrast to the resistant phenotype predicted for cytoplasmic and mitochondrial ribosomes. Interestingly, clindamycin resistance has been shown to map to the plastid genome in *Chlamydomonas* (24).

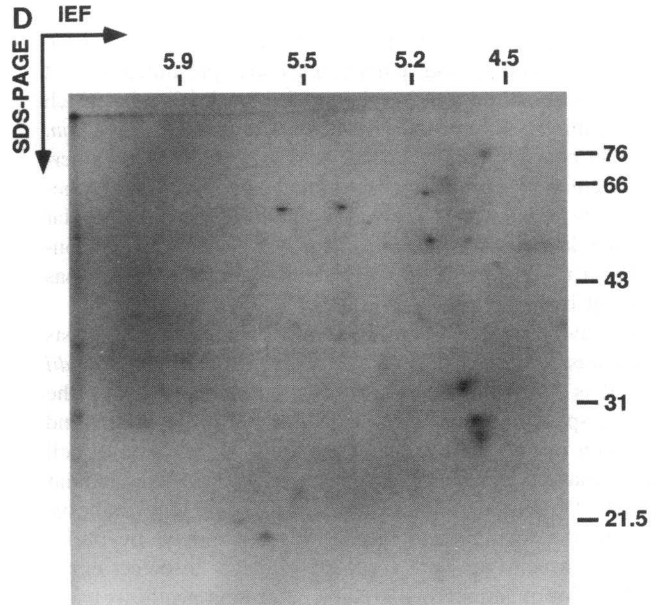
Cycloheximide-resistant (mitochondrial?) proteins are sensitive to tetracycline but not azithromycin

Because protein synthesis on both mitochondrial and plastid ribosomes is typically resistant to inhibition by cycloheximide (22, 31, 32), we sought to identify azithromycin-sensitive spots on gel electrophoretic profiles in the presence of 10 μM cycloheximide (Fig. 4). Cycloheximide treatment eliminated the vast majority of the > 300 spots seen in untreated parasites (*A*), but a characteristic pattern was retained (*B*). A few of the remaining spots (e.g., m–q in *C*) comigrate with major cycloheximide-sensitive proteins and may represent low level synthesis from cytoplasmic ribosomes, but other proteins are clearly specific to cycloheximide-resistant ribosomes. Four such spots were particularly prominent in all experiments (b [M_r = 66, PI = 5.0]; c [M_r = 64, PI = 5.35]; d [M_r = 64, PI = 5.5]; and g [M_r = 48, PI = 5.0] in *C*).









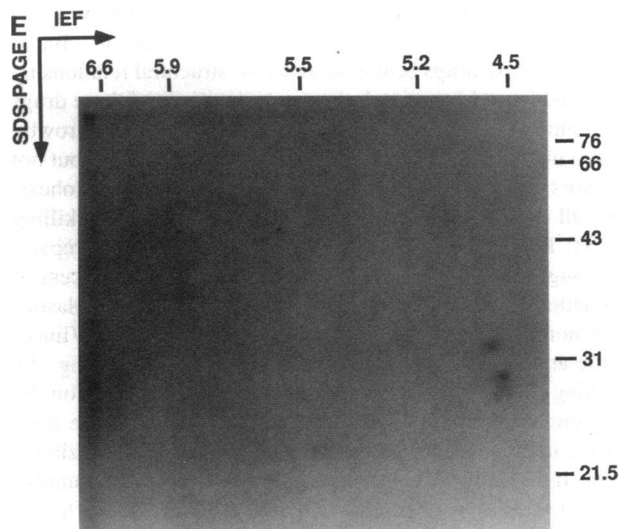


Figure 4. Susceptibility of cycloheximide-resistant protein synthesis to other antibiotics in extracellular RH train tachyzoites. (*A*) Control. (*B*) 10 μM cycloheximide. (*C*) Cartoon with designations for cycloheximide resistant proteins in (*D*) 10 μM cycloheximide + 100 μM azithromycin. (*B*) 10 μM cycloheximide + 100 μM tetracycline. (*A*) was developed overnight at room temperature; *B*, *D* and *E* were developed for 1 wk at −80°C.

Azithromycin at concentrations up to 100 μM had only minimal effect on the pattern and intensity of cycloheximide-resistant spots (*D*). Similarly, clindamycin had no reproducible effect on the cycloheximide-resistant spots (not shown). Some variability in PI was noted for several proteins migrating at the extremes of the isoelectric focusing gel (see spots h–1 in *D*), but these differences were not consistently observed. In contrast to the effects of macrolide antibiotics, 100 μM tetracycline blocked the synthesis of all cycloheximide-resistant spots detected (*E*).

To the extent detectable given the high background from host cell proteins (compare Fig. 2), the sensitivity of parasite-specific proteins to cycloheximide alone or combined with azithromycin or tetracycline in infected cells was comparable to that seen for the extracellular parasites examined in Fig. 4 (data not shown). In conjunction with the molecular sequence data in Fig. 3, the pattern of cycloheximide resistance coupled with resistance to azithromycin and sensitivity to tetracycline treatment suggests that the cycloheximide-resistant spots in Fig. 4 *B* are synthesized on mitochondrial ribosomes.

Discussion

As well-characterized inhibitors of protein synthesis, macrolides, lincosamides, and tetracyclines are presumed to act against ribosomal targets, but the precise mechanism by which these antibiotics inhibit the growth of *T. gondii, Plasmodium,* and other coccidian parasites has been the subject of considerable speculation. Combining careful mapping of protein synthesis profiles in the presence of various antibiotics with molecular sequence data on parasite ribosomal RNA genes, we have constructed a unifying hypothesis for antibiotic action which has important implications for drug design.

We have recently demonstrated that a functional pore exists in the parasitophorous vacuole membrane surrounding *T. gondii* (21). It is therefore likely that the ionic composition of the vacuolar space is equivalent to that of the host cytoplasm, and incubation in buffers mimicking the ionic composition of cell cytosol markedly enhanced protein synthesis in extracellular parasites. Despite the improved efficiency of protein synthesis, however, the total protein profile was not altered by buffer composition, and we were completely unable to detect either qualitative or quantitative alterations in protein synthesis using macrolide antibiotics, even at very high concentrations (Fig. 1). The use of host cells defective in protein synthesis (reviewed in [33]) and other recently developed techniques (34) may permit more careful analysis of intracellular parasites, but to a first approximation it appears that cytoplasmic ribosomes are not inhibited by macrolides or lincosamides. This conclusion is supported by primary sequence information from the parasite's cytoplasmic ribosomal genes (Fig. 3), which by analogy to point mutations known to confer drug resistance in other ribosomes (23, 24), predict macrolide/licosamide resistance.

We identified ~ 19 proteins whose synthesis was not completely suppressed by 10 μM cycloheximide (Fig. 4 *C*). Some of these are likely to be synthesized on noncytoplasmic ribosomes, but none is sensitive to macrolide antibiotics. In addition to the cytoplasmic ribosomal RNA genes encoded within the nuclear genome, *T. gondii,* like other coccidian parasites (35), appears to contain at least two additional classes of ribosomal RNA genes, encoded by the mitochondrial genome (present as a tandemly repeated 6-kb linear sequence in *Plasmodium* species [26]), and a plastid-like genome (a 35-kb circular molecule; [9, 29, 36, 37]). At this stage it is not possible to directly link the cycloheximide-resistant spots with either the mitochondrion or the plastid-like genomes, but the observation that the *Plasmodium* mitchondrial DNA is much more heavily transcribed than the 35-kb plastid-like circle (Vaidya, A., personal communication) argues in favor of a mitochondrial origin (38). While the functional mitochondrial genome has not yet been cloned from *Toxoplasma,* both the *P. falciparum* mitochondrial genome and sequences suspected to be derived from the *T. gondii* mitochondrial genome are available for examination, and both of these sequences predict that mitochondrial ribosomes in the phylum Apicomplexa are resistant to macrolide and licosamide antibiotics. In contrast, LSU genes identified on plastid-like genomes of both malaria and *Toxoplasma* are consistent with sensitivity to these drugs.

Tetracyclines bind most avidly to 30S ribosomes in bacteria, and doxycycline exhibits activity against *T. gondii* both in vitro and in vivo (39). In *E. coli,* multiple binding sites per ribosome (~ 300 tetracycline molecules/ribosome) have been demonstrated (40). Direct binding studies show that ~ 14 tetracycline molecules bind per ribosome for *T. gondii* cytosolic ribosomes. Indirect evidence suggests that tetracycline mediates activity against *Plasmodium* species by inhibiting mitochondrial protein synthesis (41–44), and *P. falciparum* proteins resistant to cycloheximide can be inhibited by tetracycline (42), as we have also found for *Toxoplasma* (Fig. 4). Tetracyclines may inhibit protein synthesis on other noncytoplasmic ribosomes as well (such as those encoded by the 35-kb circle), but the fact that concentrations of tetracycline in the range of 5–10 μg/ml are needed to inhibit parasite growth contrasts with the exquisite sensitivity of the parasites to nanomolar concentrations of macrolides and lincosamides. The failure to inhibit cycloheximide resistant proteins even using very high concentrations of azithromycin or clindamycin also argues that the targets for tetracycline and macrolides/lincosamides may be nonoverlapping. Recent experiments with *T. gondii* failed to detect any effect of clindamycin or azithromycin on parasite oxygen consumption, further arguing against the action of these drugs on mitochondria (3).

The failure to observe a direct effect of azithromycin or clindamycin on any parasite protein raises the spector that these drugs might be mediating microbicidal activity through some mechanism other than inhibition of protein synthesis. This possibility seems unlikely for at least three reasons, however: (*a*) azithromycin-resistant parasites are cross-resistant to clindamycin, yet these drugs bear essentially no structural relationship to one another, (*b*) extremely low concentrations of these drugs (low nanomolar) are needed for inhibition of parasite growth, and (*c*) macrolides, tetracyclines, and chloramphenicol, but not inhibitors of cytoplasmic protein synthesis such as cycloheximide, all exhibit a distinctive delayed effect on parasite killing ([2, 3]; Modica, M., and D. S. Roos, manuscript in preparation), suggesting a similar mechanism of action. By process of elimination, we propose that ribosomes encoded by the plastid-like genome represent the functional target for macrolide/lincosamide antibiotics. This hypothesis is clearly testable, e.g., by searching for proteins encoded by open reading frames on the 35-kb circle (presumably synthesized on the plastid-like ribosomes), in the presence and absence of clindamycin or azithromycin. It is likely that proteins synthesized on these putative macrolide-sensitive ribosomes are not produced in sufficient

amounts to be visualized under the conditions of our assay. Therefore, specific antibody probes to open reading frames from the *T. gondii* 35-kb circle must be developed to substantiate the hypothesis.

The availability of drug-resistant parasite mutants (3) may prove useful in directly elucidating the mechanism of macrolide/lincosamide action against *Toxoplasma.* Five mechanisms for resistance are known from bacterial studies (reviewed in [23, 45]): (*a*) induction of an RNA methylase which dimethylates adenine corresponding to base 2058 in the *E. coli* 23S subunit (macrolide-lincosamide-streptogramin B resistance) (46); (*b*) alterations in ribosomal proteins (47), (*c*) mutations in the LSU of rRNA (48, 49), (*d*) inactivation of the antibiotics, and (*e*) pump-mediated drug efflux (50). Although mechanism (*a*) is probably the most frequent in clinical resistance, mutational mechanisms are often isolated in the laboratory, and point mutations within LSU (*c*) are particularly common in lincosamide resistance (23, 24). By two-dimensional gel analysis, we have observed two new spots in an azithromycin-resistant parasite (3), which could theoretically correspond to a methylase, an altered ribosomal protein, or an efflux pump (data not shown). Uptake and retention of azithromyin is unaltered in this mutant, however, arguing against an efflux mechanism (*e*). As pointed out by Pfefferkorn and Borotz (3), it is interesting to note that the retention of clindamycin sensitivity in the azithromycin-resistant mutant resembles mutant *E. coli* strains in which uracil replaces cytosine at LSU position 2611.

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