Chapter 1

Introduction and Review of the Literature.

EPIDEMIOLOGY OF *MYCOBACTERIUM AVIUM*

Mycobacterium avium is an opportunistic pathogen of AIDS patients, infecting 25-50% of patients in the United States, Europe, and Australia (Nightingale et al., 1992; Dawson, 1990). Symptoms of *M. avium* disseminated infection include malaise, weight loss, dizziness, vomiting, diarrhea, and night sweats (Wolinsky, 1992). Patients with *M. avium* bacteremia typically have between 10^1 and 10^9 colony forming units of *M. avium* per ml of blood (Hawkins et al., 1986). *M. avium* also causes pulmonary disease in non-AIDS patients, although infrequently (1 in 100,000) (Wolinsky, 1992). One proven route of infection is through water sources in patients' environments, including tap water (von Reyn et al, 1994), as water is a natural source of *M. avium* (Falkinham et al., 1980; von Reyn et al, 1993). DNA fingerprinting studies have recently matched *M. avium* strains isolated from patients with *M. avium* strains isolated from nearby soil, adding more evidence that the environment is the primary infecting source (Via et al, submitted). Patients with AIDS and *M. avium* infection are expected to live an average of two years less than their counterparts without *M. avium* infection (Horsburgh and Seik, 1989).

POLYCLONAL *M. AVIUM* INFECTIONS.

Approximately 15 to 40% of AIDS patients with *M. avium* bacteremia are infected with more than one strain (Dawson, 1990; Arbeit et al., 1993). Using fingerprinting methods such as pulsed-field gel electrophoresis, it has been shown that polyclonal infections involving multiple strains appeared to persist for long periods in these patients (Slutsky et al., 1994) Further, studies have shown that members of polyclonal populations often differed in individual antibiotic susceptibilty (von Reyn et al., 1995). Thus, standard susceptibility testing may not be indicative of theraputic regimens that will be effective against all coexisting strains.

ANTIBIOTIC THERAPY FOR *Mycobacterium avium* DISEASE.

Treatment of disseminated *M. avium* infections in AIDS patients has proven to be problematic. One of the difficulties in treating *M. avium* infections is the relative drug-resistance of these hydrophobic, thick-walled bacteria (Rastogi et al., 1981). *M. avium* is resistant to traditional antimycobacterial drugs used in tuberculosis treatment, such as isoniazid, streptomycin, and rifampin (Rastogi et al., 1981). However, the new macrolide clarithromycin (CLA), and the azalide, azithromycin (AZI), have been shown to be effective in treatment of *M. avium* infection in AIDS patients (Dautzenberg et al., 1991; Ruf et al., 1992; Dautzenberg et al., 1993). Further, minimal inhibitory concentrations (MICs) correlated with therapeutic response (Mazur, 1993). Treatment of disseminated *M. avium* infection has usually employed a cocktail of antibiotics, including azithromycin or clarithromycin, ethambutol, and rifabutin (Gordon et al., 1993; Hoffner et al., 1994; Kissinger et al., 1995). Recent studies have shown that AIDS patients receiving antimycobacterial treatment increased their median survival from 145 days to 255 days (Ives et al., 1995). Further, patients receiving either azithromycin or clarithromycin in their treatment regimen had a median survival of 285 days compared with 168 days for patients not treated with a macrolide (Ives et al., 1995).

MACROLIDE ANTIBIOTICS.

Macrolide antibiotics are bacteriostatic agents that inhibit protein synthesis by binding to a common region on the 50S ribosomal subunit and preventing translocation. The binding site involves domain V of the 23S rRNA and several ribosomal proteins, including L4, L15, L16, and L22 (Pestka, 1977). Evidence also suggests that secondary effects of macrolides may be degradation of 50S ribosomal subunits (Vester and Garrett, 1987) and inhibition of ribosome assembly (Chittum and Champney, 1993). The macrolides share a common structure responsible for ribosome binding: an aminodeoxy sugar in glycosidic linkage to a macrolide lactone (Neu, 1991). The macrolides are lipid-soluble and enter bacterial cells by passive diffusion through the cytoplasmic membrane. Clarithromycin and azithromycin are two semisynthetic derivatives of erythromycin. Clarithromycin (Figure 1.1) is a macrolide similar to erythromycin, differing by a single substitution of a methyl group for a 6-hydroxyl group in the 14-membered erythronilide ring of the erythromycin base (Abbott Laboratories, North Chicago, IL). Azithromycin (Figure 1.2) is an azalide, differing from erythromycin by the addition of nitrogen in the erythronilide ring, resulting in a 15-member derivative (Pfizer, Groton, CT).

MECHANISMS OF MACROLIDE-RESISTANCE IN BACTERIA.

The best-characterized mechanisms of macrolide resistance in bacteria focus primarily on erythromycin. Erythromycin resistance mechanisms can be divided into two categories: modification of the target (i.e. the 50S ribosomal subunit), and modification of the antibiotic. Examples of target modification include methylation of 23S rRNA, mutations in 23S rRNA genes, and mutations in ribosomal protein genes. Examples of antibiotic modification include active efflux, inactivation, and cell permeability changes.

The primary mechanism of resistance in clinically important bacteria, such as Gram-positive cocci, is methylation of 23S rRNA (Lai and Weissblum, 1971). Such resistance is induced by low levels of erythromcyin, and confers resistance to macrolides, licosamides, and streptogramin B (MLS) antibiotics. Resistance via this mechanism is primarily plasmid-encoded, and involves a member of the *erm* gene family. *Erm* genes, thirteen of which have been characterized, encode a methylase that dimethylates position 2058 or 2059 in the 23S rRNA (Arthur et al., 1990).

Mutations in ribosomal proteins associated with the 50S subunit have also been described in erythromycin-resistant mutants. Early studies with erythromycin-resistant *Escherichia coli* showed that ribosomal proteins L4 and L22 from resistant strains exhibited different migration patterns than their sensitive counterparts in two-dimensional gels (Otaka et al., 1970; Wittman et al., 1973). Later studies, in which the genes from each mutant were sequenced, found a point mutation in the *rplD* gene (encoding ribosomal protein L4) of one strain, causing an amino acid replacement of lysine to glutamic acid at position 63. In the second strain, a three codon deletion in the *rplV* gene (encoding ribosomal protein L22) was discovered, leading to loss of the sequence methionine-lysine-arginine at residues 82-84 in protein L22 (Chittum and Champney, 1993).

Mutations in the 23S rRNA sequence itself have also been investigated as mechanisms of erythromycin resistance. Mutations have been characterized in the large ribosomal RNA in the mitochondria of *Saccharomyces cervisiae* which correspond to position 2058 in domain V in the 23S rRNA of *E. coli* (Sor and Fukuhara, 1982). Erythromycin resistance in *E. coli* has also been localized to a 23S rRNA gene on a multi-copy plasmid containing a cloned rRNA operon (*rrnH*). Erythromycin-resistant clones were isolated following transformation of susceptible cells with the plasmid. Deletion of the 23S rRNA gene from the construct restored sensitivity, whereas deletion

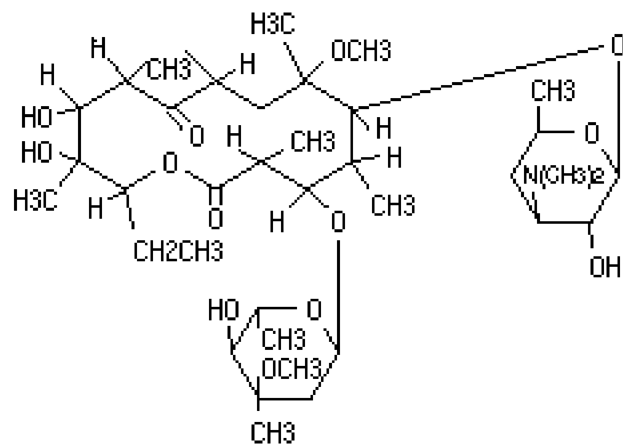


Figure 1.1. Structure of clarithromycin (Abbott Laboratories, Chicago, IL).

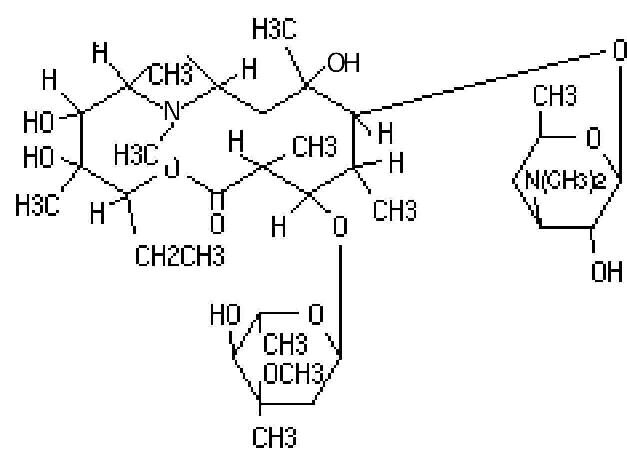


Figure 1.2. Structure of azithromycin (Pfizer, Groton, CT).

of the 16S rRNA gene did not (Sigmund and Morgan, 1982). Further, site-directed mutagenesis introducing an A to G transversion at position 2058 in a plasmid-encoded 23S rRNA gene in *E. coli* conferred erythromycin resistance when the plasmid was transformed into a sensitive background (Vester and Garrett, 1987). These studies suggested that mutations in ribosomal RNA leading to erythromycin resistance are either dominant or co-dominant, at least when located on a multi-copy plasmid (Sigmund and Morgan, 1982; Vester and Garrett, 1987). It has also been demonstrated that mutations in domain II of the 23S rRNA in *E. coli* interact with and alter the conformation of domain V, leading to erythromycin resistance (Douthwaite and Aagaard, 1993)

Inactivation of erythromcyin has been demonstrated as a mechanism of erythromycin resistance in several organisms. In *Streptomyces vendargensisi*, erythromycin is glycosylated, resulting in 2'-(O-[ß-D-glucopyranosyl]) erythromycin, which is inactive (Kuo et al., 1989). Inactivation by glycosylation or phosphorylation has also been described in pathogenic *Nocardia* species (Yazawa et al., 1994). In certain strains of *E. coli*, inactivation of oleandomycin has been demonstrated, resulting in the isolation of an altered compound, oleandomycin 2'-phosphate. It was subsequently shown that this inactivation was the result of an inducible intracellular enzyme, macrolide 2' phosphotransferase (O'Hara et al., 1989). Phosphorylation has also been described in *Streptomyces coelicolor*, whereby the macrolides oleandomycin, spiramycin, tylosin, and leucomycin A were enzymatically phosphorylated, producing inactive macrolide 2'-*O*-phosphates (Marshall et al., 1989). Enzymatic hydrolysis of erythromycin has also been reported as a mechanism of resistance in *E. coli*. This resistance is the result of a plasmid-encoded esterase that hyrolyzes the lactone ring of the macrolide (Barthelemy et al., 1984).

An additional mechanism of macrolide resistance described in the literature is active efflux. Certain resistant strains of *Staphylococcus epidermidis* possess a plasmid-encoded transmembrane protein responsible for the active efflux of erythromycin (Ross et al., 1990). This type of resistance is inducible, and appears to work in concert with other efflux pathways in the cells (Ross et al., 1990).

MACROLIDE RESISTANCE IN *Mycobacterium avium*.

Although clarithromycin and azithromycin are essential components of combination therapy for *M. avium* infection, there have been reports of relapse and the emergence of clarithromycin-resistant (Cla^R) and azithromycin-resistant (Azi^R) *M. avium* isolates from AIDS patients undergoing treatment with clarithromycin or azithromycin alone (Dautzenberg et al., 1991; Ruf et al., 1992; Dautzenberg et al., 1993). The rate of treatment failure in those patients was approximately 25%. *M. avium* isolates exhibiting high-level resistance (>64 µg/ml) are resistant to both azithromycin and clarithromycin. In addition, spontaneous macrolide resistance in *M. avium* occurs at a frequency of 1×10^{-9} (Heifets et al., 1993).

Recently, some mechanisms of resistance to clarithromycin and azithromycin in *Mycobacterium avium* have been elucidated. The most common mechanism of resistance, accounting for 80% of high-level resistant isolates (MIC >64 µg/ml), is mutation of a specific residue, A-2058, in the single copy 23S rRNA gene (Meier et al., 1994; Nash and Inderleid, 1995; Jensen-Cain and Falkinham, 1997) . In approximately 10% of high-level resistant isolates, resistance is associated with a mutation at position A-2059 in the 23S rRNA gene (Meier et al., 1994; Nash and Inderleid, 1995; Jensen-Cain and Falkinham, 1997). It has been hypothesized

that mutations at A-2058 or A-2059 alter the conformation of the ribosome and prevent macrolide binding (Nash and Inderleid, 1995). The mechanism(s) of resistance in the other 10% of high-level resistant isolates remains unknown, as do mechanism(s) of intermediate-level resistance to azithromycin and clarithromycin.

Specific Objectives

1. Determine the heritage of macrolide- and azalide-resistant clinical isolates of *Mycobacterium avium* using DNA fingerprinting.

2. Determine whether high-level resistance results from methylation of rRNA, mutations in the peptidyl transfer region of the 23S rRNA gene, or other mutations affecting the ribosome.

3. Determine the effect of clarithromycin and azithromycin on amino acid incorporation in cell-free systems from macrolide-sensitive and -resistant strains.

4. Determine whether intermediate resistance results from inactivation, active efflux, or changes in permeability of the cell wall or cell membrane.

Chapter 2

Submitted as: **D.M. Jensen-Cain, L.E. Via, C.A. Benson, A.S. Vorys, and J.O. Falkinham, III.** Origin of Macrolide-Resistant Isolates of *Mycobacterium avium* in AIDS Patients Undergoing THerapy. *Submitted to:* Antimicrob. Agents Chemother.

Origin of Macrolide-Resistant Isolates of *Mycobacterium avium* in Patients Undergoing Therapy

ABSTRACT

Although clarithromycin and azithromycin have been effective in prophylaxis and treatment of *M. avium* bacteremia in AIDS patients, resistance has emerged in patients during single drug therapy with these agents. Two different DNA fingerprinting methods, random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) of the insertion sequence IS 1245, have been employed to determine the heritage of azithromycin and clarithromycin-resistant *M. avium* isolates recovered from individual patients. Comparison of fingerprints from pre-treatment (sensitive) and post-treatment (resistant) isolates from seven separate patients showed that resistance resulted from spontaneous mutation of the original, azithromycin and clarithromycin-sensitive isolate in five of the seven patients. In the other two patients, the clarithromycin-resistant isolates were unrelated to the sensitive isolate, suggesting that the resistant isolate resulted from either superinfection or selection of a resistant strain from a polyclonal population.

INTRODUCTION

Mycobacterium avium is an opportunistic pathogen responsible for disseminated infection in 25-50% of patients with AIDS. *M. avium* also causes the most common systemic infection in patients with AIDS in the United States (Horsburgh and Seik, 1989; Nightingale et al., 1992; Benson and Ellner, 1993). Treatment of *M. avium* bacteremia is problematic, as the organism is inherently resistant to most antimycobacterial agents (Rastogi et al., 1981). To further complicate treatment, between 15-40% of *M. avium*-infected AIDS patients appear to be infected with more than one strain (Dawson, 1990; Arbeit et al., 1993). Von Reyn et al. report that strains from mixed populations often differ in their susceptibility to antibiotics (1995). If only single isolates from such patients are subjected to drug susceptibility testing, antibiotics chosen for therapy based on the minimal inhibitory concentrations (MICs) of one isolate may be ineffective in treating the whole mixed population.

Clarithromycin (Abbott Laboratories, North Chicago, IL) is a macrolide similar to erythromycin, differing by a single substitution of a methyl group for a 6-hydroxyl group in the 14-membered erythronilide ring of the erythromycin base (Benson et al., 1987). Azithromycin (Pfizer, Groton, CT) is an azalide, differing from erythromycin by the addition of nitrogen in the erythronilide ring, resulting in a 15-member derivative (Inderleid, 1989). Azithromycin and clarithromycin, primarily in combination with other antibiotics, have been shown to be effective in treating *M. avium* infections (Dautzenberg et al., 1991; Ruf et al., 1992; Husson et al., 1994). Azithromycin and clarithromycin are also approved for prophylaxis of *M. avium* infection in AIDS patients (Havlir et al., 1996). However, *M. avium* isolates resistant to both azithromycin and clarithromycin have been recovered from patients undergoing single-drug treatment (Dautzenberg et al., 1991; Heifets et al., 1993; Husson et al., 1994). It is possible these drug-resistant isolates could be spontaneous mutants of the original sensitive strain. Although spontaneous mutation conferring azalide or macrolide resistance occurs at a low frequency (approximately 1×10^{-9} drug-resistant colony forming units per total population), the large total number of *M. avium* cells present in infected AIDS patients (Heifets et al., 1993) make the appearance of mutants likely. Alternatively, mutant isolates could represent minor (and undetected), azalide- or macrolide-resistant members of a polyclonal population that were selected for during therapy, or could be due to superinfection with another drug-resistant *M. avium* strain.

In order to determine the heritage of drug-resistant isolates recovered from individual patients during therapy with azithromycin or clarithromycin, we chose to use DNA fingerprinting to compare pre-treatment (sensitive) and post-treatment (resistant) isolates from these patients. The rationale for this approach was based on previous studies demonstrating that DNA fingerprint patterns of *M. avium* isolates from different AIDS patients are quite diverse (Arbeit et al., 1993; Mazurek et al., 1993). Therefore, fingerprints of sensitive and resistant isolates from a single patient would be similar if azalide or macrolide resistance was the result of spontaneous mutation, yet different if the resistant strain was a minor, undetected member of the original population or if the patient was infected by another drug-resistant *M. avium* strain. The first method tested to compare the isolates was random amplified polymorphic DNA (RAPD) fingerprinting . RAPD fingerprinting is a PCR-based method that utilizes single 10-base primers that bind to target DNA and generate a banding pattern specific for a given strain (Williams et al., 1990). RAPD profiles can thus be compared to determine the similarity between strains (Welsh and McClelland, 1990; Wang et al., 1993). DNA hybridizations using probes generated from RAPD bands can be used to further confirm similarity of bands (Williams et al., 1990). The second method tested was restriction fragment length polymorphism (RFLP) of the insertion sequence IS1245 described by Guerrero et al. (1995). Members of the *M. avium* complex contain multiple copies of this

transposable element (Guerrero et al., 1995) and genetic relatedness can be determined by comparison of banding patterns following hybridization of genomic DNA digests with an IS1245 probe.

MATERIALS AND METHODS

***Mycobacterium avium* strains.** *M. avium* strains 201-1 and 201-2 and *M. avium* strains 214-1 and 214-2 are pre-treatment and post-treatment isolates, respectively, from two AIDS patients with *M. avium* bacteremia enrolled in a dose-ranging clarithromycin treatment trial (Chaisson et al., 1994). *M. avium* strains 306-1, 306-2, 306-3, and 306-4, are pre-treatment (306-1) and post-treatment (306-2, 306-3, and 306-4) isolates recovered at successive intervals from one patient with AIDS and *M. avium* bacteremia treated with azithromycin. *M. avium* strains 504 and 511, 505 and 512, and 506 and 513 are clinical isolates from three HIV-infected pediatric patients treated with clarithromycin for *M. avium* infection (Nash and Inderleid, 1995). *M. avium* strains 9010-1, 9010-2, and 9010-3 are isolates from a non-AIDS patient treated with clarithromycin for MAC pulmonary disease. All post-treatment isolates were recovered from patients following relapse and treatment failure.

Growth of strains. Mycobacteria strains were inoculated into 10 ml Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco, Detroit, MI) and incubated at 37˚C without shaking for 2 weeks.

Minimal inhibitory concentrations (MICs). The minimal inhibitory concentrations of clarithromycin and azithromycin were determined for each isolate using a broth microdilution assay (Inderleid, 1990). Serial two-fold dilutions in Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, MD), pH 7.4, containing 10% (vol/vol) OADC were made in microtiter plates starting with a 25 µg/ml concentration of either clarithromycin or azithromycin, leaving one row as a no antibiotic control. Each well was inoculated with 2.5 µl of each *M. avium* strain suspended in buffered saline-gelatin solution (0.85% NaCl, 0.01% gelatin in 6.8 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.0) adjusted to a McFarland standard of 0.5, and incubated for 7-10 days at 37˚C. The MICs were the lowest concentration of antibiotic at which there was no visible growth after 10 days. For those strains resistant to 25 µg/ml of either clarithromycin and/or azithromycin, the experiment was repeated, starting with a concentration of 100 µg drug/ml.

DNA isolation. DNA was isolated from cells harvested from unshaken cultures grown at 37˚C for two weeks using the procedure of Via and Falkinham (1994). The cells were disrupted with glass beads in the Mini Bead-Beater (Biospec Products, Bartelsville, OK), and DNA was extracted with a 10% (wt/vol) hexadecyltrimethylammonium bromide (CTAB) in 0.7 M NaCl solution following treatment with DNase-free RNase and Proteinase K (Sigma Chemical Company, St. Louis, MO). The aqueous and organic phases were separated using chloroform-isoamyl alcohol (24:1), and the DNA precipitated from the aqueous phase with 100% (vol/vol) isopropanol. DNA pellets were washed with 70% (vol/vol) ethanol, and dissolved in TE after drying. DNA concentrations were measured spectrophotometrically at 260 nm and all DNAs diluted to 2 ng/µl in UV-irradiated sterile distilled water.

RAPD reactions. RAPD reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA). Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.3 mM each deoxyribonucleotide, 50 pmoles of a single 10-base oligonucleotide primer, 2% (vol/vol) glycerol, 1 unit *Taq* DNA polymerase (Promega, Madison, WI), and 6 ng of template DNA. Each reaction was subjected to an initial denaturation for 5 min at 95˚C, followed by 75 cycles of 94˚C for 10s , 36˚C for 10s with a ramp of 0.2˚C/s to 46˚C, and 72˚C for 2 min. The amplification products were separated by gel electrophoresis (3V/cm) through a 1.8% gel

containing 1.0% (wt/vol) agarose and 0.8% (wt/vol) Synergel^R (Diversified BioTech, Newton Centre, CT) in recirculating TAE buffer (Sambrook et al., 1989). DNA bands were visualized by ethidium bromide staining. The sequences of the 10-base RAPD primers (Operon Tech, Inc., Alameda, CA) are listed in Table 2.1.

Generation of IS 1245 probe. The IS 1245 sequence was amplified by PCR using primers IS1245-F and IS1245-R (Table 2.1) designed from the GenBank sequence (Accession number L33879). Each reaction contained 50 mM KCl, 10mM Tris-HCl (pH 8.3), 1.7 mM $MgCl_2$, 0.3 mM each deoxyribonucleotide, 30 pmol of each oligonucleotide primer, 1 unit *Taq* DNA polymerase, and 10 ng of template DNA. The reactions were subjected to an initial denaturation for 5 min at 95˚C, followed by 30 cycles of 94˚C for 1 min, 58˚C for 1 min, and 72˚C for 2 min. The resulting product was purified using PrepAGene (BioRad, Hercules, CA), and a probe was produced by random primed labeling with digoxigenin-conjugated dUTP using the Genius DNA Hybridization System as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN).

Southern blotting. Genomic DNA (1 µg) was digested with either *Eco*RI or *Sal*I (Promega, Madison, WI) overnight at 37˚C. Following agarose gel electrophoresis for 3 hours at 5 V/cm in an 0.8% agarose gel, the DNA was transferred to a nylon membrane by alkaline blotting overnight in 0.4 N NaOH following depurination in 0.3 N HCl for 5-10 min. The resulting blots were hybridized with probe at a concentration of 10 ng per ml of hybridization buffer (5X SSC, 0.01% sodium dodecyl sulfate, 0.5% n-lauroyl sarcosine, and 1% Boehringer Mannheim blocking reagent) at 65˚C for 18 h. Unbound probe was washed from the membrane at room temperature with an initial 2 min wash in 2X SSC, 0.5% SDS, followed by a 20 min wash in the same solution, and two 20 min washes in 2X SSC, 0.1% SDS. The membrane was rinsed in 2X SSC, and bound probe was detected using CSPD®, a chemiluminescent substrate, as described by the manufacturer (Boehringer Mannheim, Indianapolis, IA).

Band sharing calculation. In order to compare banding patterns of two or more strains generated by either method, the total number of bands present in the compared fingerprints were counted, as well as the number of bands common to all compared strains. Percent band sharing was determined by dividing the number of common bands by the number of total bands.

Table 2.1. Sequences of oligonucleotide primers.

Primer	Sequence
OPA-13	5' CAGCACCCAC 3'
OPB-7	5' GGTGACGCAG 3'
OPI-20	5' AAAGTGCGGG 3'
IS1245-F	5' TCCGCCGTGAGTCTCTGTGGTGAAA 3'
IS1245-R	5' AGAGCGGCTGTGGGGGCAATGGTT 3'

RESULTS AND DISCUSSION

MICs of azithromycin and clarithromycin. The MICs of azithromycin and clarithromycin for each isolate are presented in Table 2.2. The pre-treatment isolates had MICs ranging from 0.1 - 0.4 µg clarithromycin per ml and 0.8 - 6.25 µg azithromycin per ml. Six of the ten post-treatment isolates had MICs greater than 100 µg/ml for both clarithromycin and azithromycin. In contrast, post-treatment strains 306-2, 306-3, and 306-4 had MICs between 0.1 - 5 µg clarithromycin/ml, and 40 - 60 µg azithromycin/ml. Post-treatment strain 9010-2 had an MIC of 16 µg clarithromycin/ml. These results suggest that emergence of resistance was the reason for the relapse and treatment failure in the patients.

RAPD analysis of patient isolates. Figures 2.1, 2.2, and 2.3 show the PCR products amplified with primers OPA-13, OPB-07, and OPI-20, respectively. The percent band sharing among isolates is presented in Table 2.3. Note that only isolates 214-1 and 214-2, and 505 and 512 have a percent band sharing below 30%. This suggests that the emergence of resistance in patients 201, 306, 504, 506, and 9010 was the result of spontaneous mutation of the original sensitive *M. avium* isolate, whereas the emergence of resistance in patients 214 and 505 appeared to be the result of proliferation by an unrelated macrolide-resistant isolate of *M. avium*. The results with strains 505 and 512 are consistent with those using pulsed-field gel electrophoresis of large restriction fragments in previous work (Nash and Inderleid, 1995).

Hybridization of selected bands to RAPD profiles. In order to show that amplification products that migrated the same distance in the gel represented homologous DNA fragments, one RAPD band generated from an individual isolate was recovered from a low-melting point agarose gel, labeled, and subsequently used as a hybridization probe against the rest of the bands generated with that primer. Probes were made from the 1.1 kb band from strain 214-1 DNA generated with primer OPA-13 (Fig. 2.1), the 1.2 kb band from strain 201-1 DNA generated with primer OPB-07 (Fig. 2.2), and the 1.4 kb band from strain 306-3 DNA generated with primer OPI-20 (Fig. 2.3). The results of these experiments are shown in Figure 2.4. Each probe hybridized with equal-sized bands that migrated the same distance on its RAPD profile, indicating that amplification products that migrated the same distance were homologous. In addition, each probe also hybridized uniformly to smaller amplification products, suggesting that internal primer sites may exist within these larger bands, or that RAPD primers are amplifying similar DNA sequences from different regions of the bacterial chromosome.

RFLP analysis of patient isolates. Figures 2.5 and 2.6 show the autoradiographs resulting from hybridization of Southern blots with IS1245. The percent band sharing among isolates is presented in Table 2.3. Note that only isolates 214-1 and 214-2, and 505 and 512 have a percent band sharing below 94%. These results confirm the conculsion reached in the RAPD fingerprinting studies that the emergence of resistance in patients 201, 306, 504, 506, and 9010 was the result of spontaneous mutation of the original sensitive *M. avium* isolate, whereas the emergence of resistance in patients 214 and 505 appeared to be the result of proliferation by an unrelated macrolide-resistant isolate of *M. avium*. These results also agree with fingerprinting data for strains 505 and 512 using pulsed-field gel electrophoresis of large restriction fragments (Nash and Inderleid, 1995).

Azalide or Macrolide-resistant *M. avium*. This study has led us to make several observations regarding the emergence of azithromycin- or clarithromycin-resistant *M. avium* isolates in AIDS patients undergoing antibiotic therapy. Based on the large numbers of *M. avium* organisms found in blood and infected tissues in patients with AIDS and disseminated *M. avium*

Table 2.2. Minimal inhibitory concentrations (MICs) of azithromycin and/or clarithromycin for *M. avium* isolates.

M. avium strain	MIC of Cla (µg/ml)	MIC of Azi (µg/ml)
201-1	0.8	3.12
201-2	>100	>100
214-1	0.8	6.25
241-2	>100	>100
306-1	0.1	8
306-2	0.2	38
306-3	4	38
306-4	5	60
504	0.1	nd
511	>100	nd
505	<5	nd
512	>100	nd
506	0.2	nd
513	>100	nd
9010-1	<5	nd
9010-2	16	nd
9010-3	>100	nd

nd = not done

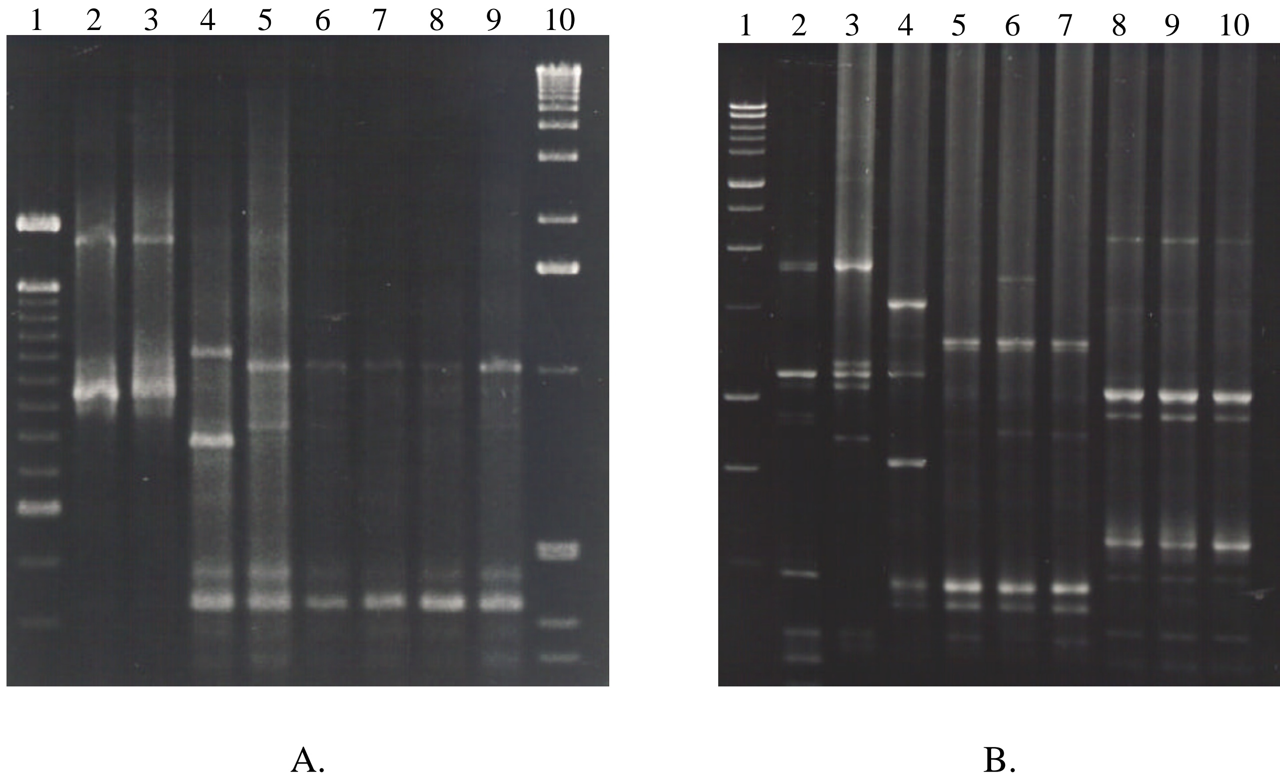


Figure 2.1 *M. avium* RAPD profiles generated with primer OPA-13.

Panel A: Lanes 1 and 10, size markers (100 bp ladder and 1 kb ladder, Gibco BRL, Gaithersburg, MD); lane 2, 201-1; lane 3, 201-2; lane 4, 214-1; lane 5, 214-2; lane 6, 306-1; lane 7, 306-2; lane 8, 306-3; lane 9, 306-4.

Panel B: Lane 1, size marker (1 kb ladder, Promega, Madison, WI); lane 2, 504; lane 3, 511; lane 4, 505; lane 5, 512; lane 6, 506; lane 7, 513; lane 8, 9010-1; lane 9, 9010-2, lane 10, 9010-3.

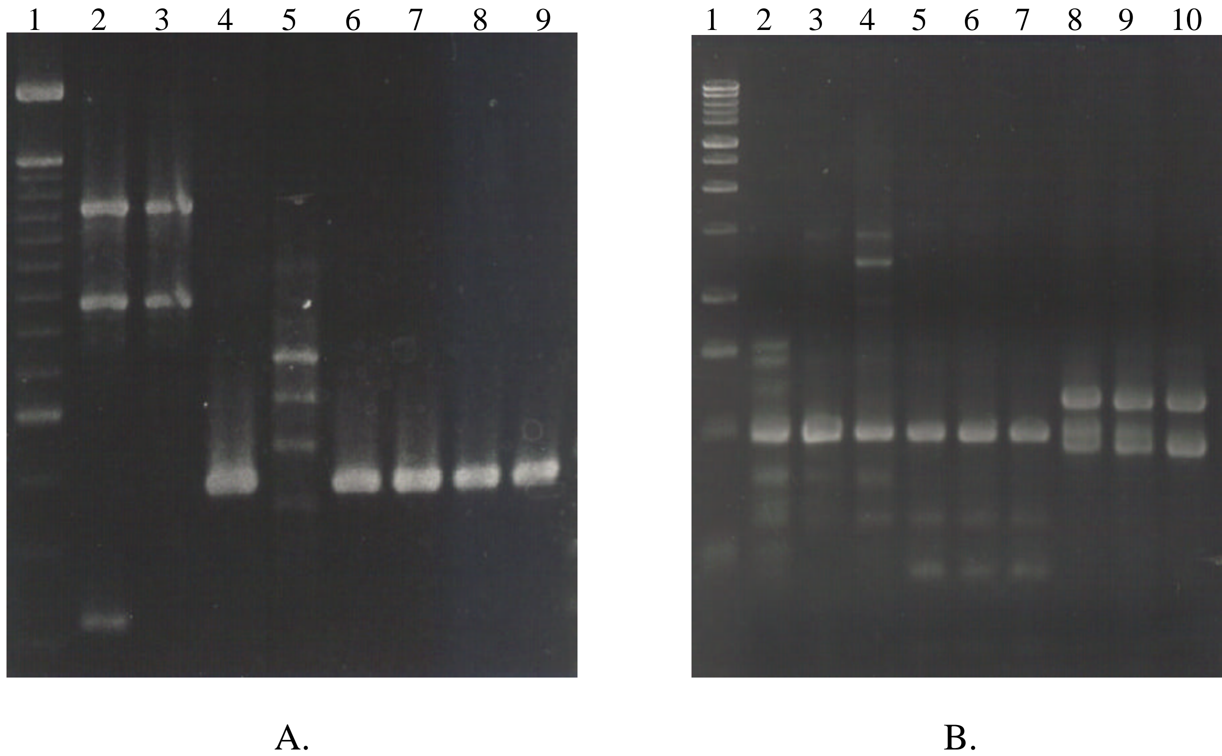


Figure 2.2. *M. avium* RAPD profiles generated with primer OPB-07.

Panel A: Lane 1, size marker (100 bp ladder, Gibco BRL, Gaithersburg, MD); lane 2, 201-1; lane 3, 201-2; lane 4, 214-1; lane 5, 214-2; lane 6, 306-1; lane 7, 306-2; lane 8, 306-3; lane 9, 306-4.

Panel B: Lane 1, size marker (1 kb ladder, Promega, Madison, WI); lane 2, 504; lane 3, 511; lane 4, 505; lane 5, 512; lane 6, 506; lane 7, 513; lane 8, 9010-1; lane 9, 9010-2, lane 10, 9010-3.

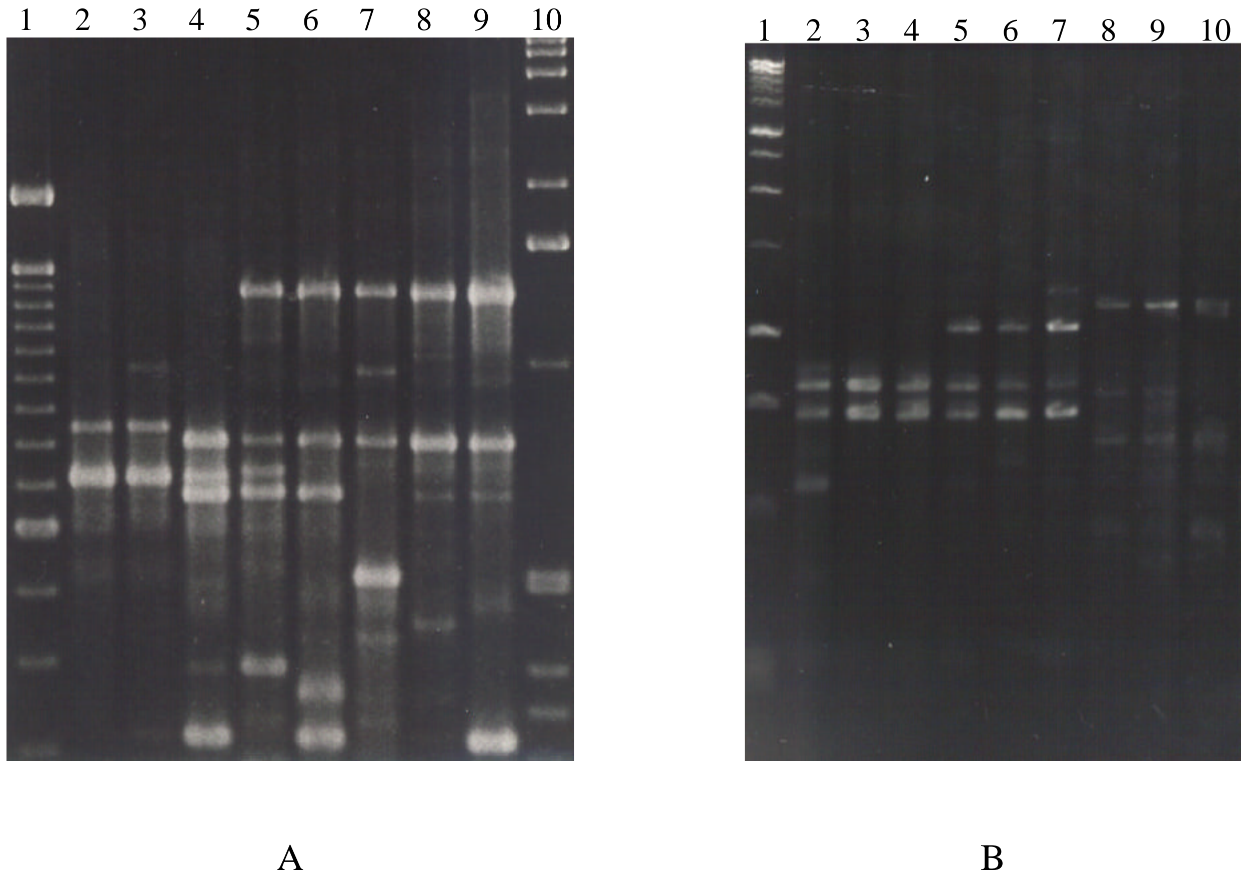


Figure 2.3. *M. avium* RAPD profiles generated with primer OPI-20.

Panel A: Lanes 1 and 10, size markers (100 bp ladder and 1 kb ladder, Gibco BRL, Gaithersburg, MD); lane 2, 201-1; lane 3, 201-2; lane 4, 214-1; lane 5, 214-2; lane 6, 306-1; lane 7, 306-2; lane 8, 306-3; lane 9, 306-4.

Panel B: Lane 1, size marker (1 kb ladder, Promega, Madison, WI); lane 2, 504; lane 3, 511; lane 4, 505; lane 5, 512; lane 6, 506; lane 7, 513; lane 8, 9010-1; lane 9, 9010-2, lane 10, 9010-3.

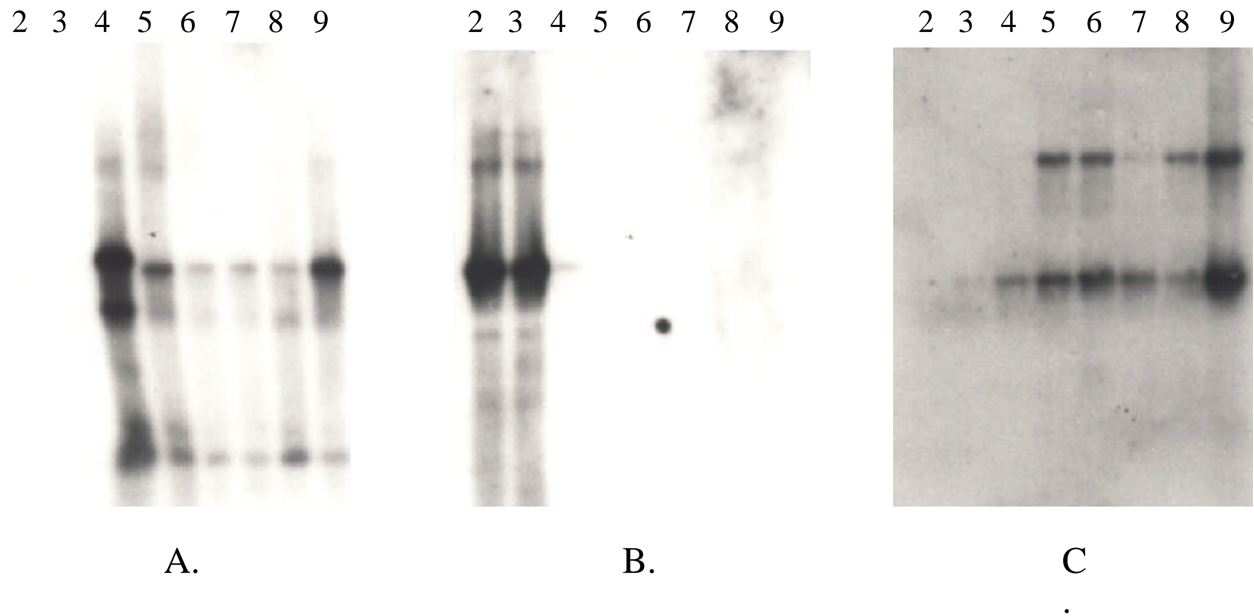


Figure 2.4. Southern blot analysis of *M. avium* RAPD profiles.

Lane 2, 201-1; lane 3, 201-2; lane 4, 214-1; lane 5, 214-2; lane 6, 306-1; lane 7, 306-2; lane 8, 306-3; lane 9, 306-4.

Panel A: The gel shown in Fig. 2.1 was blotted and hybridized with the 1.1 kb band from strain 214-1 generated with primer OPA-13.

Panel B: The gel shown in Fig. 2.2 was blotted and hybridized with the 1.2 kb band from strain 201-1 generated with primer OPB-07.

Panel C: The gel shown in Fig. 2.3 was blotted and hybridized with the 1.4 kb band from strain 306-3 generated with primer OPI-20.

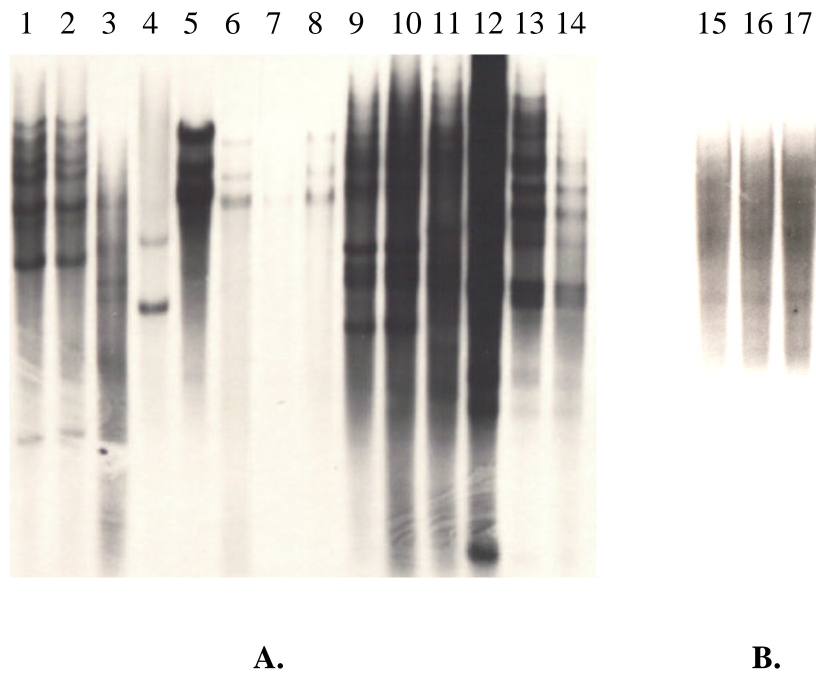


Figure 2.5. Southern blot of *M. avium* genomic DNA digested with *Eco*RI and hybridized with IS1245 probe.

Lane 1, 201-1; lane 2, 201-2; lane 3, 214-1; lane 4, 214-2; lane 5, 306-1; lane 6, 306-2; lane 7, 306-3; lane 8, 306-4, lane 9, 504; lane 10, 511; lane 11, 505; lane 12, 512; lane 13, 506; lane 14, 513; lane 15, 9010-1; lane 16, 9010-2; and lane 17; 9010-3.

(Lanes 1-14 were exposed to film for 30 minutes after application of CSPD. Lanes 15-17 required an 80 minute exposure after application of CSPD.)

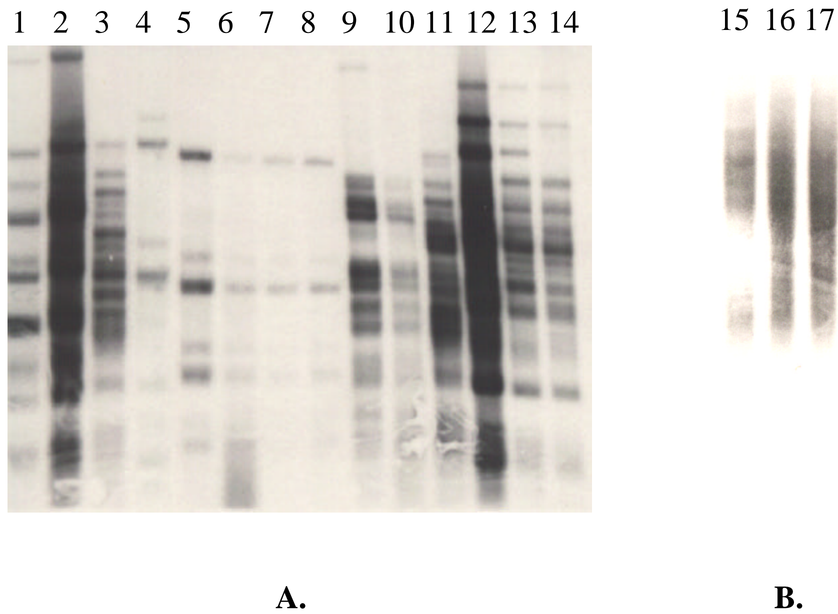


Figure 2.6. Southern blot of *M. avium* genomic DNA digested with *Sal*I and hybridized with IS1245 probe.

Lane 1, 201-1; lane 2, 201-2; lane 3, 214-1; lane 4, 214-2; lane 5, 306-1; lane 6, 306-2; lane 7, 306-3; lane 8, 306-4, lane 9, 504; lane 10, 511; lane 11, 505; lane 12, 512; lane 13, 506; lane 14, 513; lane 15, 9010-1; lane 16, 9010-2; and lane 17; 9010-3.

(Lanes 1-14 were exposed to film for 30 minutes after application of CSPD. Lanes 15-17 required an 80 minute exposure after application of CSPD.)

Table 2.3. RAPD and RFLP Analysis of Macrolide-sensitive and Subsequent Macrolide-resistant *Mycobacterium avium* Isolates from Infected Patients.

M. avium strain	RAPD: % Band Sharing	IS1245 RFLP: % Band Sharing
201-1		
201-2	78% (7/9)	100% (17/17)
214-1		
214-2	23% (5/22)	10% (2/19)
306-1		
306-2		
306-3		
306-4	64% (7/11)	100% (9/9)
504		
511	56% (9/16)	94% (17/18)
505		
512	29% (4/14)	8% (3/38)
506		
513	85% (11/13)	95% (18/19)
9010-1		
9010-2		
9010-3	90% (9/10)	100% (12/12)

complex disease (Heifets et al., 1993), resistant isolates can be expected to result from spontaneous mutation of a susceptible strain, as was seen with patients 201, 306, 504, 506, and 9010. However, the data from patients 214 and 505 suggests the emergence of a resistant isolate was due to another mechanism, since the resistant isolate from this patient was unrelated to the initial susceptible isolate. The resistant isolate could have originated through superinfection of the patient after treatment successfully eradicated the initial infecting strain. This scenario is not unlikely, since AIDS patients are readily infected by *M. avium* (Horsburgh and Seik, 1989). However, inherent macrolide resistance among isolates recovered from patients or the environment in the absence of prior exposure to a macrolide or azalide has thus far been infrequently described (Chaisson et al., 1994). The source, then, of such a superinfecting strain remains speculative. Alternatively, the resistant isolate could have been a member of a polyclonal population which was selected for during therapy; that is, either this unrelated isolate was initially sensitive and it mutated and responded to selection during treatment, or it was initially resistant and was thus selected.

This study underscores the importance of recognizing the presence of polyclonal infections with *M. avium* in patients with AIDS and of considering the potential impact of differential drug susceptibility of these strains on the outcome of treatment. Pre-treatment susceptibility to clarithromycin has been shown to be associated with positive clinical and microbiologic response to treatment (Dautzenberg et al., 1991; Young et al., 1991; Ruf et al., 1992; Dautzenberg et al., 1993; Chaisson et al., 1994). Thus far, this relationship has been demonstrated only for this agent in patients with AIDS. The data in this report, however, suggests that pre-treatment antibiotic susceptibility testing for *M. avium* complex may be more predictive of treatment efficacy if susceptibility testing is performed on more than one isolate.

This study also offers a direct comparison of three fingerprinting methods for *Mycobacterium avium*: random amplifed polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) of the insertion sequence IS 1245, and pulsed-field gel electrophoresis of large restriction fragments. The results with strains 505 and 512 demonstrate that both RAPD and RFLP fingerprinting are as discriminatory as pulsed-field gel electrophoresis of large restriction fragments. In addition, this study suggests that RAPD fingerprinting offers the possibility of a greater range of discrimination than the other two methods, allowing distinction between strains that are closely related.

Chapter 3

Submitted as: **D.M. Jensen-Cain and J.O. Falkinham, III.** Identification of Clarithromycin-Resistant *Mycobacterium avium* Isolates Using the Polymerase Chain Reaction. *Submitted to:* J. Clinical Microbiol.

Identification of mutations in the 23S rRNA gene associated with macrolide resistance in *Mycobacterium avium*, and detection of macrolide-resistant *M. avium* isolates using PCR.

ABSTRACT

Clarithromycin (CLA) and azithromycin (AZI) have been effective in treating or preventing *Mycobacterium avium* bacteremia in AIDS patients. However, resistance has emerged in patients undergoing single drug therapy. We have used PCR to determine that macrolide resistance does not appear to be due to an *Erm*-like methylation system in *M. avium.* Further, we have used single-track dideoxy sequencing to confirm that high-level macrolide resistance (>64 µg/ml) in some strains results from a point mutation at position 2058 in the 23S rRNA. In contrast, strains exhibiting intermediate resistance had no mutations in the peptidyl transfer region of the 23S rRNA. From these results, a rapid PCR-based screen for clarithromycin-resistance in *Mycobacterium avium* has been developed. Using a degenerate 20-nucleotide primer targeted to the peptidyl transfer region of the 23S rRNA gene, PCR reactions using an annealing temperature of 69˚C resulted in a 250 bp product only if a point mutation has occurred at position 2058. Twenty-three clinical isolates were analyzed using this method, seven of which were CLA^R. The 250 bp product was amplified only in CLA^R strains, indicating those strains had mutations at 2058, which is consistant with sequence data.. This method was also able to detect a CLA^R subpopulation in a polyclonal population of *M. avium*.

INTRODUCTION

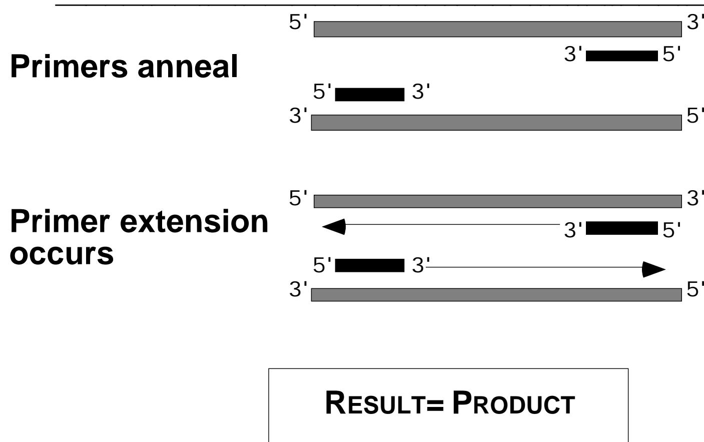
The macrolide, clarithromycin (Abbott Laboratories, Chicago, IL) and the azilide, azithromycin (Pfizer, Groton, CT), are protein synthesis inhibitors that, in combination with other antibiotics, have been shown to be effective in treating *Mycobacterium avium* infections. However, *M. avium* isolates resistant to azithromycin and clarithromycin have been recovered from patients undergoing single-drug therapy (Chaisson et al., 1994). Resistance to macrolide antibiotics has been shown to result from point mutations at a specific position, A-2058, in the 23S rRNA of *Escherichia coli* (Sigmund and Morgan, 1982), *Mycobacterium intracellulare* (Meier et al., 1994), and the mitochondrial DNA of *Saccharomyces cervisiae* (Sor and Fukuhara, 1982); or by plasmid-encoded methylation of this residue in *Staphylococcus aureus* (Lai and Weisblum, 1971), other Gram-positive cocci, and *E. coli* (Courvalin, 1986)

In order to identify the mechanisms for resistance in *M. avium*, a collection of *M. avium* antibiotic-sensitive clinical isolates, and subsequent clarithromycin- and azithromycin-resistant isolates that emerged during clarithromycin or azithromycin therapy of the same patients, were examined for changes in the 23S rRNA. First, degenerate primers specific for members of the *erm* rRNA methylase gene family were used to determine if macrolide- and azalide-resistant *M. avium* isolates contained *erm*-like rRNA methylase genes (Arthur et al., 1990). Second, we have examined the isolates for mutations at A-2058 using single-track dideoxy sequencing with only ddATP in the chain termination reactions. Thus, strains with point mutations at A-2058 could be readily identified by the absence of a band, then fully sequenced to confirm the mutation.

PCR-based methods have been demonstrated effective in detection of antibiotic-resistance in many organisms, most notably with Gram-positive cocci to detect resistance to macrolides (Arthur et al., 1990) methicillin, and vancomycin (Aarestrup et al., 1996). The use of rapid, PCR-based detection methods with slowly growing organisms such as *M. avium* offers the advantage that small numbers of organisms can be detected by DNA amplification, bypassing the need for long-term culturing and providing antibiotic-susceptibility data in a short time.

In this study, we have also developed a PCR-based method for detecting macrolide-resistant isolates of *M. avium* that have mutations at residue A-2058 in the 23S rRNA gene. The method is based on the principle that oligonucleotide primers cannot be extended if there is a mismatch at the 3' end of the primer (Figure 3.1). Using this method, one class of CLA^R *M. avium* isolates can be quickly identified by the amplification of a 250 bp product.

When NO MISMATCH is present at 3' end of primers:



When a MISMATCH is present at 3' end of one primer:

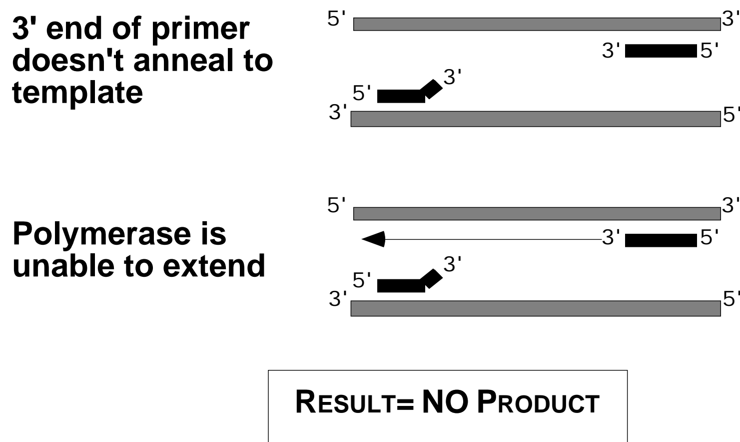


Figure 3.1. PCR detection of point mutations.

MATERIALS and METHODS.

Isolates and MICS. *M. avium* strains 112, 201-1, 201-2, 206, 214-1, 214-2, and 217 are isolates from AIDS patients with *M. avium* bacteremia enrolled in a dose-ranging clarithromycin treatment trial (Chaisson et al., 1994). *M. avium* strains 503, KN503, 504, 505, 506, 507, 508, 510, 511, KN511, 512, 513, and 514 are clinical isolates from HIV-infected pediatric patients treated with CLA for *M. avium* infection (Nash and Inderleid, 1995). *M. avium* strains 9010-1, 9010-2, and 9010-3 are isolates from an HIV-negative patient treated with clarithromycin for MAC pulmonary disease (von Reyn, personal communication). *M. avium* strains 306-1, 306-2, 306-3, and 306-4 are from an HIV-infected patient treated with azithromycin. Susceptibility to clarithromycin and azithromycin was determined for each isolate using a broth microdilution assay (Inderleid, personal communication), in which serial two-fold dilutions of antibiotic in Mueller-Hinton broth (BBL Microbiological Systems, Cockeysville, MD) with 10% OADC (BBL Microbiological Systems, Cockeysville, MD), pH 7.4, were made in microtiter plates. Wells were inoculated with 2.5 µl of *M. avium* culture adjusted to a McFarland standard of 0.5, and incubated without shaking for 7-10 days at 37˚C. The MICs were the lowest concentration of antibiotic at which there was no visible growth.

DNA isolation. DNA was isolated from *M. avium* cultures grown 7-10 days at 37˚C in Middlebrook 7H9 broth (BBL Microbiological Systems, Cockeysville, MD) containing 0.5% glycerol and 10% OADC. Cells were harvested by centrifugation (5,000 x g at 4˚C for 10 min) and disrupted 1 min with 0.1 mm glass beads in the Mini Bead-Beater® (Biospec Products, Bartelsville, OK). The DNA was extracted with a 10% (wt/vol) hexadecyltrimethylammonium bromide (CTAB) in 0.7 M NaCl solution following treatment with DNase-free RNase and Proteinase K (Via and Falkinham, 1994). The aqueous phase was extracted using chloroform isoamyl alcohol (24:1) and the DNA precipitated from the aqueous phase with 100% (vol/vol) isopropanol. DNA pellets were washed with 70% (vol/vol) ethanol and dissolved in TE buffer (Sambrook et al., 1989) after drying. DNA concentrations were measured by spectrophotometry, and all DNAs were diluted to 2 ng/µl in UV-irradiated sterile distilled water for use in PCR.

Screening for rRNA methylase genes. Degenerate primers (Table 3.1) specific for members of the rRNA methylase gene-family were used to determine if macrolide-resistant *M. avium* isolates contained rRNA methylase genes in the *Erm* family (Arthur et al., 1990). Each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.7 mM $MgCl_2$, 0.3 mM each deoxyribonucleotide, 60 pmol of each oligonucleotide primer (ERM1 and ERM2), 1 unit *Taq* DNA polymerase, and 6 ng of template DNA. Each reaction was subjected to an initial denaturation for 3 min at 95˚C, followed by 35 cycles of 94˚C for 30 s, 42˚C for 2 min, and 72˚C for 90 s (Arthur et al., 1990). DNA isolated from a rRNA methylase-producing strain of *Enterococcus faecalis* was used as a positive control. The amplification products were separated by gel electrophoresis (4V/cm) through a 1 % agarose gel. DNA bands were visualized by ethidium bromide staining.

Sequencing. Domain V of the 23S rRNA gene was amplified by PCR in 90 µl volumes, cleaned with the Prep-A-Gene DNA purification kit (BioRad, Richmond, CA), and eluted in 40 µl elution buffer. The PCR products were directly sequenced using the amplification primers with Sequenase 2.0 according to the manufacturer's instructions (US Biochemical, Cleveland, OH). All strains were screened for mutations at A-2058 by sequencing using only ddATP in the chain termination reactions. Following single-track sequencing of all strains, the peptidyl transfer region of the 23S rRNA gene from resistant strains was PCR-amplified in 90 µl volumes, and the PCR products were directly sequenced using the amplification primers with Sequenase 2.0 (US Biochemical, Cleveland, OH) to determine the point mutation present at position 2058.

Table 3.1. Sequences of oligonucleotide primers.

Primer	Sequence
ERM1	5' G A R A G I G I I I I G G I A A R G G I C A 3'
ERM2	5' A A Y T G R T T Y T T I G T R A A 3'
FG1	5' A A T G G C G T A A C G A C T T C T C A 3'
FG2	5' C T T T G G G C G C C T C C G T T A C A T 3'
23SV-WT	5' G T T A C G C G C G G C A G G A C G A A 3'
23SV-WT2	5' G T T A C G C G C G G C A G G A C G C A 3'
23SV-WT3	5' G T T A C G C G C G G C A G G A C T A A 3'
CLA-R	5' G T T A C G C G C G G C A G G A C G A B 3'

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; I, inosine, R, adenine and guanine; Y, cytosine and thymine; B, cytosine, guanine, and thymine.

PCR detection of A-2058 mutations. A forward primer, CLA-R, was designed with the 3' end degenerate for C, G, and T at position 2058 in the 23S rRNA. Primers CLA-R and FG2 were used to detect mutations as described in the introduction. Primers FG1 and FG2 were used to amplify domain V for sequencing. Primer 23SV-WT is identical to CLA-R, with the exception that its 3' end is an A. Primer sequences are listed in Table 3.1.

The optimal annealing temperature at which the sequence was amplified in resistant strains but not sensitive strains was determined using DNA from strains 201-1, 201-2 and 214-2. The PCR reactions were performed in a Robocycler (Stratagene, San Diego, CA) in which the annealing block was programmed with a temperature gradient varying 2˚C from 60 to 74˚C. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.7 mM $MgCl_2$, 0.3 mM each deoxyribonucleotide, 30 pmol of each oligonucleotide primer (CLA-R and FG2), 1 unit *Taq* DNA polymerase, and 10 ng of template DNA. Each reaction was subjected to an initial denaturation for 5 min at 95˚C, followed by 30 cycles of 94˚C for 1 min, T_m for 1 min, and 72˚C for 2 min. The amplification products were separated by gel electrophoresis (4V/cm) through a 2% agarose gel. DNA bands were visualized by ethidium bromide staining.

PCR screen of clinical *M. avium* isolates for A-2058 mutation. Twenty-three clinical isolates were analyzed, seven of which were CLA^R and AZI^R, using primers CLA-R and FG2. The reactions were performed as described above, with an annealing temperature of 70˚C.

Three artificially mixed populations of *M. avium* strains were screened for CLA resistance. Population A contained equal numbers (determined by absorbance at 580 nm) of four *M. avium* strains (201-1, 203, 504, and 214-2), one of which was CLA^R (214-2). Population B contained a mixture of the same four *M. avium* strains, in which the CLA^R strain was present in 1/10 the concentration of the other strains. Population C contained a mixture of the same four *M. avium* strains, in which the CLA^R strain was present in 1/100 the concentration of the other strains. Population D contained an equal mixture of three *M. avium* strains, all of which were CLA^S. Strains were grown individually for 3 to 4 days to equivalent turbidities, and mixed to yield populations A - D. DNA was isolated from one ml of a 3 - 4 day old culture by suspending the cell pellet in 200 µl distilled water, subjecting the suspension to lysis with 0.1 mm glass beads for 80 s in the Mini Bead-Beater®, and centrifuging the lysate for 5 min at 10,000 x g. 5 µl of the supernatant was used in the PCR reactions which were performed as described above.

Primer specificity. In order to determine if mismatches at positions close to the 3' end of the primer would also prevent amplification, variants of primer 23SV-WT were synthesized. One had a mismatch at position 2057 (primer 23SV-WT2) and the second had a mismatch at 2056 (primer 23SV-WT3). These primers were used with genomic DNA from a sensitive *M. avium* strain, 201-1, in a PCR reaction with a temperature gradient as described above.

RESULTS

Macrolide resistance and 23S rRNA alterations. *M. avium* strains 201-2, 214-2, 511, KN511, 512, 513, 514, and 9010-3 were all found to be resistant to high levels (>64 µg/ml) of clarithromycin. *M. avium* strain 9010-2 exhibited intermediate resistance to clarithromycin (16 µg/ml) and strains 306-2, 306-3, and 306-4 exhibited intermediate resistance to azithromycin (40 - 60 µg/ml). Using primers ERM1 and ERM2, specific for methylase genes, in PCR reactions, DNA from all macrolide-sensitive and -resistant *M. avium* strains failed to amplify, although the *E. faecalis* control did show a 500 bp band. However, DNA from all *M. avium* strains was amplified using genus-specific primers, assuring the integrity of the template DNA. Thus, it appears that rRNA methylation via an *erm*-like mechanism did not confer macrolide-resistance in these *M. avium* strains.

Initial sequence data from high-level resistant (>64 µg/ml) *M. avium* strains 201-2 and 214-2, compared with their sensitive counterparts, showed mutations at position 2058 in the 23S rRNA gene (Figure 3.2). *M. avium* strains 306-2, 306-3, and 306-4 had no mutations in the peptidyl transfer region of the 23S rRNA. Upon sequencing all sensitive and resistant strains, the sequence data from the peptidyl transfer region of the 23S rRNA in these strains identified an A at position 2058 and 2059 in all sensitive strains, and a C or G at position 2058 in six of the seven high-level resistant strains. The seventh resistant strain, 9010-3, had a C at position 2059 (Table 3.2) . These data strongly suggest that mutations at position 2058 or 2059 in the 23S rRNA confer macrolide-resistance in *M. avium*, and are consistent with reports in the literature (Meier et al., 1994; Nash and Inderleid, 1995).

PCR detection of A-2058 mutations. The optimal annealing temperature for discrimination between CLA^R and CLA^S strains was found to be 69˚C. As shown in Figure 3.3, the 250 bp target will only be amplified from template DNA from sensitive strains at annealing temperatures at and below 66˚C, whereas the target will be amplified from template DNA from CLA^R strains at temperatures of up to 74˚C. Of the twenty-three clinical isolates analyzed using the described primers, the 250 bp product was amplified in six strains (Figure 3.4), all of which were CLA^R, and had a point mutation at A-2058, as confirmed by sequencing (Table 3.2). These results indicate that the PCR-based method is effective in detecting CLA^R *M. avium* isolates, and does not give false-positive results. The results also underscore the utility of using a degenerate primer that can detect any change at position 2058. In one CLA^R strain, 9010-3, no amplification occurred; however, this strain had a point mutation at A-2059, rather than A-2058. Thus, this assay is unable to detect the minority class of CLA^R isolates that are resistant due to A-2059 mutations. However, detection of a CLA^R member of a polyclonal population was possible even when the isolate was 0.3% of the other sensitive strains.

Mismatches at one or two bases from the 3' end of the forward primer did not prevent amplification of the 250 bp region (Figure 3.5), even at stringent temperatures, although primer-template binding was restricted to the inherent annealing temperature of individual primers. Evidently, polymerization constraints due to primer-template mismatches apply only to the terminal 3' residue. Therefore, detection methods utilizing 3' mismatches are likely to be specific for a single position, and false-negatives will not result from mismatches one or two bases from the 3' end.

Table 3.2. *M. avium* strains used in this study. Listed below are the susceptibilities of the strains to clarithromycin, their partial 23S rRNA sequence in the peptidyl transfer region, and the results of the PCR test for clarithromycin-resistance.

Strain	MIC of Cla (μg/ml)	Phenotype	23S rRNA domainV sequence	Band amplified with CLA-R primers
112	0.2	Cla^S	g g a c g a A a g a c c	-
201-1	0.8	Cla^S	g g a c g a A a g a c c	-
201-2	>100	Cla^R	g g a c g a C a g a c c	+
203	0.4	Cla^S	g g a c g a A a g a c c	-
206	0.1	Cla^S	g g a c g a A a g a c c	-
213	0.8	Cla^S	g g a c g a A a g a c c	-
214-1	0.8	Cla^S	g g a c g a A a g a c c	-
214-2	>100	Cla^R	g g a c g a C a g a c c	+
217	1.51	Cla^S	g g a c g a A a g a c c	-
9010-1	<5	Cla^S	g g a c g a A a g a c c	-
9010-3	>100	Cla^R	g g a c g a a C g a c c	-
503	0.1	Cla^S	g g a c g a A a g a c c	-
KN503	<5	Cla^S	g g a c g a A a g a c c	-
504	0.1	Cla^S	g g a c g a A a g a c c	-
505	<5	Cla^S	g g a c g a A a g a c c	-
506	0.2	Cla^S	g g a c g a A a g a c c	-
507	0.8	Cla^S	g g a c g a A a g a c c	-
508	0.4	Cla^S	g g a c g a A a g a c c	-
511	>100	Cla^R	g g a c g a C a g a c c	+
KN511	>100	Cla^R	g g a c g a G a g a c c	+
512	>100	Cla^R	g g a c g a C a g a c c	+
513	>100	Cla^R	g g a c g a G a g a c c	+
514	<5	Cla^S	g g a c g a A a g a c c	-

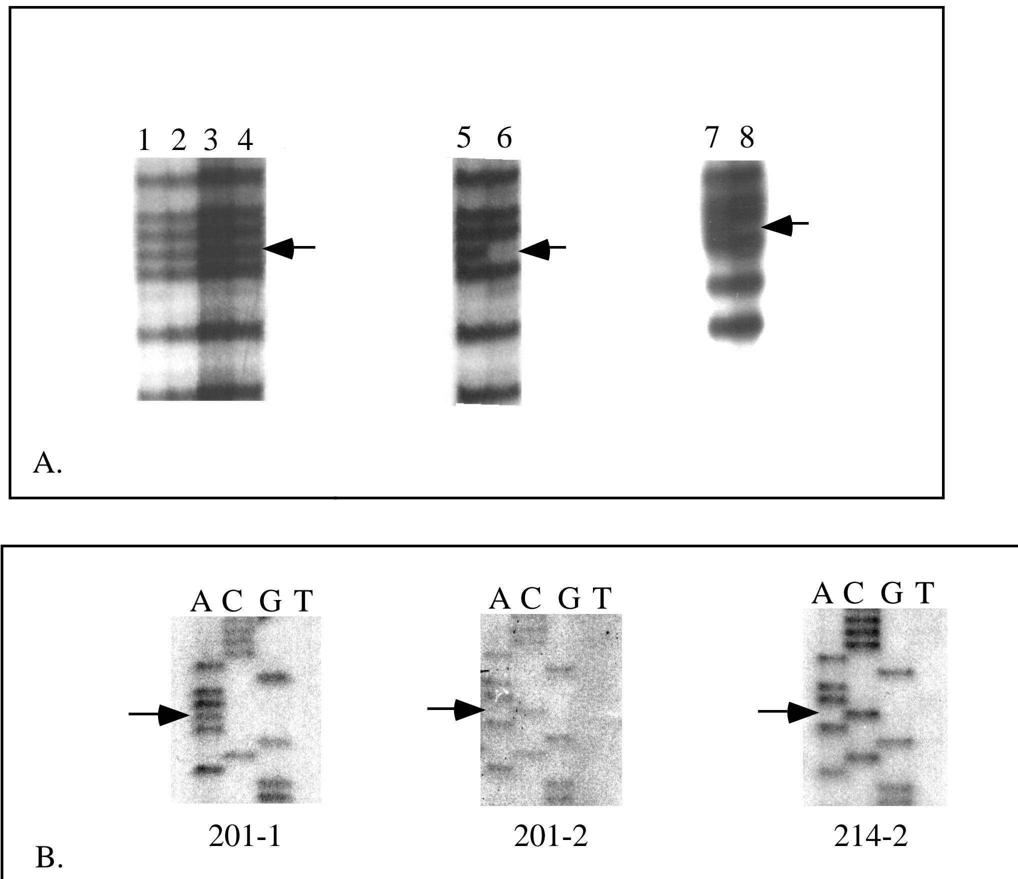


Figure 3.2. Sequence of peptidyl transfer region of 23S rRNA of *M. avium* strains.

Panel A: Single-track sequencing using only ddATP in the chain termination reactions. Lane 1, 306-1; lane 2, 306-2; lane 3, 306-3; lane 4, 306-4; lane 5, 201-1; lane 6, 201-2; lane 7, 214-1; lane 8, 214-2; Note absence of A at position 2058 in lanes 6 and 8.

Panel B: Sequence of *M. avium* strains 201-1, 201-2, and 214-2; Note A to C transversion at position 2058 in 201-2 and 214-2

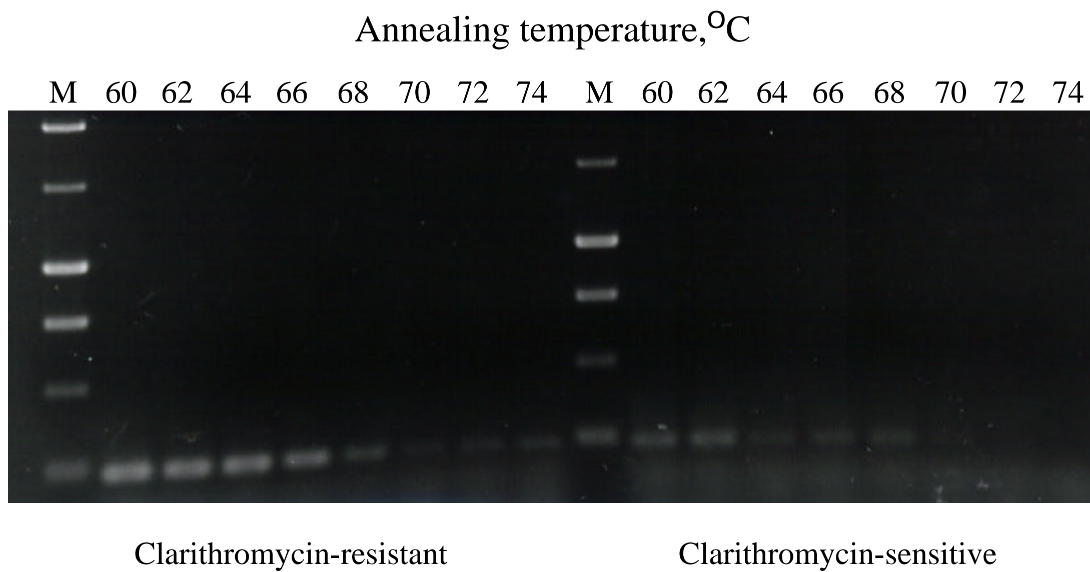
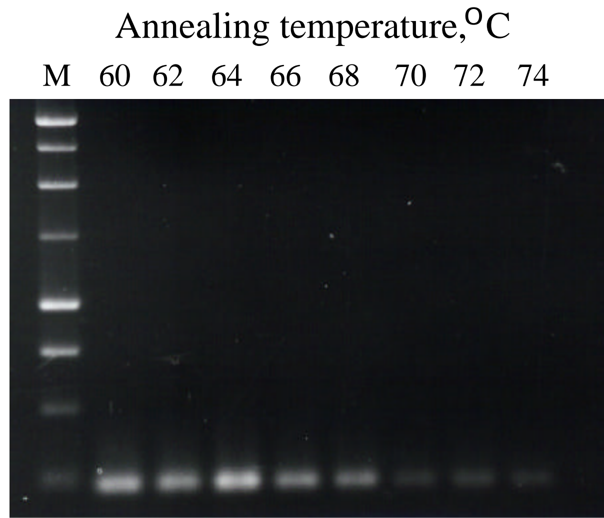
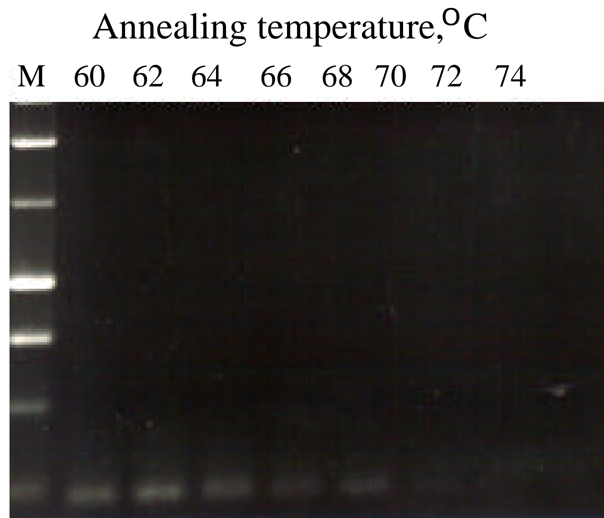


Figure 3.3. Optimization of annealing temperature for PCR detection of mutations leading to clarithromycin resistance. M, marker (1 kb ladder, Promega, Madison, WI)

Figure 3.4. PCR detection of clarithromycin resistance in clinical *M. avium* isolates using primers CLA-R and FG2.



A.



B.

Figure 3.5. Specificity of primer-template mismatches in preventing PCR amplification. Reactions were performed as described in materials and methods.

Panel A: PCR products using primers 23SV-WT2 and FG2

Panel B: PCR products using primers 23SV-WT3 and FG2

Chapter 4

Development and use of an *in vitro* translation system for characterizing macrolide resistance in *Mycobacterium avium*

ABSTRACT

A polyuridylic acid (poly U)-dependent *in vitro* translation system from *Mycobacterium avium* was developed by improving on existing protocols for *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. The optimized system was used to characterize macrolide resistance in *M. avium* clinical isolates. Clarithromycin was an effective inhibitor of protein synthesis in cell-free extracts of the susceptible *M. avium* strain, 201-1, whereas strain 201-2, which exhibited high-level resistance (>64 µg/ml), was less susceptible to clarithromycin *in vitro.* Further, equal mixtures of S-20 protein from 201-1 and 201-2 showed a pattern of clarithromycin inhibition similar to the resistant strain, suggesting that resistance may be dominant in partial diploids. In clinical isolates that showed sensitivity to clarithromycin and intermediate-level resistance to azithromycin (<64 µg/ml), the susceptibility of cell-free extracts in the *in vitro* translation system to different concentrations of azithromycin correlated with that of the susceptible parent, suggesting resistance was not a consequence of a mutation affecting the antibiotic target.

INTRODUCTION

Azithromycin (Pfizer, Groton, CT) and clarithromycin (Abbott Laboratories, Chicago, IL) are macrolide antibiotics commonly used in treatment (Dautzenberg et al., 1991; Young et al., 1991; Ruf et al., 1992) and prophylaxis (Havlir et al., 1996; Pierce et al., 1996) of disseminated *Mycobacterium avium* infection in AIDS patients, as well as in treatment of *M. avium* pulmonary disease in non-HIV infected persons. *M. avium* isolates resistant to azithromycin and clarithromycin have emerged in patients undergoing monotherapy (Ruf et al., 1992; Heifets et al., 1993). Reversion to sensitivity does not occur in the absence of antibiotic selection, suggesting that resistance is conferred by a stable mutation in a target gene or in a gene whose product modifies a target.

Macrolides are protein synthesis inhibitors that target the peptidyl transfer region of the 50S ribosomal subunit, preventing peptide chain elongation (Pestka, 1977). In organisms such as *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, macrolides have been shown to inhibit protein synthesis in cell-free extracts in *in vitro* translation assays (Wilhelm and Corcoran, 1967; Mao and Weigand, 1968; Mao and Robishaw, 1971). In addition, protein synthesis in these systems was not inhibited by macrolides using cell-free extracts from resistant strains possessing mutations in ribosomal protein genes (Wilhelm and Corcoran, 1967). As described in Chapter 3, *M. avium* strains resistant to high levels of clarithromycin and azithromycin often have a mutation at position A-2058 or A-2059 in the single-copy 23S rRNA gene. However, it has never been demonstrated that resistant organisms possessing this mutation are still able to synthesize protein in the presence of the antibiotic. In addition, *M. avium* strains have been isolated that exhibit intermediate resistance to clarithromycin and/or azithromycin and lack a mutation in the 23S rRNA gene. The mechanism of resistance in these strains is unknown.

Efficient *in vitro* translation systems have only been described for *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, and *Mycobacterium tuberculosis* (Shaila et al., 1973). In this study, we describe a cell-free system from *M. avium* capable of efficient incorporation of [^{14}C]- amino acids into protein. In addition, we describe the use of this system to characterize the effect of clarithromycin on protein synthesis in cell-free extracts from isogenic clarithromycin-sensitive and -resistant *M. avium* strains, as well as mixtures of cell-free extracts from these strains. Further, we describe the use of this system to characterize *M. avium* strains that exhibit intermediate resistance to azithromycin.

MATERIALS AND METHODS

Bacterial strains. *M. avium* strains 201-1 and 201-2 are pre-treatment and post-treatment isolates from an AIDS patient with *M. avium* bacteremia enrolled in a dose-ranging clarithromycin treatment trial (Chaisson et al., 1994). Strain 201-1 is clarithromycin-sensitive (MIC<1 µg/ml) and 201-2 is clarithromycin-resistant (MIC>100 µg/ml). Strain 201-2 has an A to C transversion at position 2058 in the 23S rRNA gene. DNA fingerprinting studies have shown that 201-1 and 201-2 are isogenic strains (Jensen-Cain et al., 1997). *M. avium* strains 306-1, 306-2, 306-3, and 306-4 are pre-treatment (306-1) and post-treatment (306-2, 306-3, and 306-4) isolates from an AIDS patient with disseminated *M. avium* infection treated with azithromycin. Strain 306-1 is azithromycin-sensitive (MIC=8 µg/ml) and successive isolates exhibit step-wise resistance (MIC=40, 40, and 60 µg/ml for 306-2, 306-3, and 306-4, respectively). All four strains are wild type at positions 2058 and 2059 in the 23S rRNA, and their intermediate resistance to azithromycin is stable in the absence of the antibiotic. DNA fingerprinting studies have shown that 306-1, 306-2, 306-3, and 306-4 are isogenic strains (Jensen-Cain et al., 1997).

Chemicals and reagents. Clarithromycin was donated by Abbott Laboratories, Chicago, IL, and azithromycin was donated by Pfizer, Inc, Groton, CT. Phosphoenol-pyruvate, pyruvate kinase, GTP, L-amino acids, *Escherichia coli* tRNA (type XX), and polyU were purchased from Sigma Chemical company (St. Louis, MO). Radiolabeled [^{14}C]-phenylalanine was purchased from Amersham (Arlington Heights, IL). Stock solutions of antibiotics were prepared in dimethyl sulfoxide at a concentration of 20 mg/ml.

Preparation of S-20 extract. *M. avium* strains were grown in 200 ml Middlebrook 7H9 broth (BBL, Cockeysville, MD) containing 0.5% (vol/vol) Tween 80 (Sigma, St. Louis, MO), 0.5% (vol/vol) glycerol, and 10% (vol/vol) ADC enrichment (Difco, Detroit, MI) for 5-7 days at 37˚C with rotary shaking at 80 rpm. Cells were harvested by centrifugation at 8,000 x g at 4˚C and washed once in chilled buffer (0.01 M Tris, pH 7.8, 0.01 magnesium acetate, 0.06 M ammonium chloride, and 0.006 M 2-mercaptoethanol, Yamada et al., 1972). The pellet was resuspended in approximately 1 ml of the same buffer, and transferred to a tube containing one gram chilled glass beads. The cells were subjected to the following disruption conditions in the Mini Bead-Beater® (Biospec Products, Bartelsville, OK) to determine conditions for obtaining maximal S-20 protein: (1) 0.5 mm glass beads for one min, (2) 0.5 mm glass beads for three min, (3) 0.1 mm glass beads for one min, (4) 0.1 mm glass beads for three min or (5) 0.1 mm glass beads for five min. Cell disruption was performed for 3 -5 min in one minute intervals, incubating on ice for one minute between each interval. The lysate was centrifuged briefly (5 min at 10,000 x g) to pellet the glass beads and cell debris, and the supernatant centrifuged at 4˚C for 45 min at 20,000 x g (Shaila et al., 1973). The resulting supernatant was the S-20 extract. The amount of S-20 protein was measured using the BioRad Protein assay with bovine serum albumin as the protein standard (BioRad, La Jolla, CA).

Assay of incorporation of [^{14}C]-phenylalanine into polypeptides. Reaction mixtures (110 µl) containing 50 mM Tris buffer, pH 7.8, 3 mM ammonium chloride, 15 mM magnesium acetate, 2 mM dithiothreitol, 10 mM phosphoenol pyruvate, 5 µg pyruvate kinase, 0.2 mM GTP, 0.1 mM each amino acid (except phenylalanine), 100 µg *Escherichia coli* tRNA (type XX), 40 to 80 µg S-20 protein, and azithromycin or clarithromycin at the appropriate concentration were pre-incubated for 10 min at 37˚C, then chilled briefly. Following the addition of 100 µg polyU and 0.05 µCi [^{14}C]-phenylalanine, incubation was continued at 37˚C for an additional 30 minutes. Samples (100 µl) were spotted onto 2.3 cm Whatman 3MM filter disks, pre-treated for 5 min with

100 µl 1N NaOH to destroy amino acyl-tRNA complexes, and allowed to stand at room temperature for 30 min (Shaila et al., 1973). Disks were dropped into 10% TCA, then washed three times in 5% TCA for 10 min each wash. The disks were then washed in an ethanol:ether mixture (3:1), and then in ether alone, and allowed to air dry. The amount of [^{14}C]-phenylalanine incorporation was assayed by scintillation counts using ScintiSafe (Fisher, Pittsburgh, PA) scintillation fluid.

RESULTS and DISCUSSION.

Lysis conditions. Conditions for lysis yielding highest amounts of S-20 protein from *M. avium* were determined to be 3 min of cell disruption in the Mini-bead beater using 0.1 mm glass beads (Table 4.1). Disruption for 3 or 5 min with 0.1 mm glass beads yielded the most protein, followed by disruption for 3 min with 0.5 mm glass beads. Disruption for 1 min with either 0.1 mm or 0.5 mm glass beads did not allow recovery of sufficient S-20 protein (Table 4.1).

Kinetics of Incorporation. [^{14}C]-phenylalanine incorporation was linear up to 30 minutes (Figure 4.1). A 5- to 10- fold stimulation in reactions to which polyU was added was observed over the blank containing no polyU.

Concentration of tRNA. As previously reported with *M. tuberculosis* (Shaila et al., 1973), cell-free extracts from *M. avium* strains appear to be limiting in endogenous tRNA (Figure 4.2). Thus, it was necessary to add high amounts of *E. coli* tRNA to the system. Reactions (110 µl) containing either 25 µg, 50 µg, 100 µg, or 150 µg tRNA showed that 100 µg tRNA was suitable for high levels of [^{14}C]-phenylalanine incorporation (Figure 4.2).

Effect of azithromycin and clarithromycin on [^{14}C]-phenylalanine incorporation. Both azithromycin and clarithromycin inhibited [^{14}C]-phenylalanine incorporation in cell-free extracts from *M. avium* strains 306-1 (AZI^S) and 201-1 (CLA^S). As shown in Figure 4.3, inhibition increased with increasing antibiotic concentration. Inhibition by azithromycin and clarithromycin was pH-sensitive, having an optimal pH for inhibition of 7.4 - 7.8 (data not shown). This is in agreement with MIC data, as MICs with *M. avium* are much lower at pH 7.4 than 6.8 (Heifets et al., 1992), presumably due to the fact that high-pK macrolides are less active when protonated. At pH values below 7.4, phenylalanine incorporation was not sensitive to azithromycin, and was in fact stimulated by azithromycin below pH 6.8, which is consistent with reports in the literature for other high-pK macrolides such as erythromycin and oleandomycin (Mao and Weigand, 1968).

As shown in Figure 4.4, [^{14}C]-phenylalanine incorporation in cell-free extracts from the high-level (>64 µg/ml) resistant strain, 201-2, was much less sensitive to clarithromycin inhibition than the sensitive strain, 201-1. This data is in agreement with the MIC data, and provides further evidence that mutations at A-2058 in the 23S rRNA gene of *M. avium* do confer macrolide-resistance by preventing macrolide inhibition of protein synthesis. Interestingly, [^{14}C]-phenylalanine incorporation in equal mixtures of S-20 protein from 201-1 (sensitive) and 201-2 (resistant) exhibited resistance to clarithromycin similar to that of the resistant strain, 201-2 (Figure 4.5). This suggests that in partial diploids, macrolide resistance is dominant to sensitivity, which is in agreement with reports in the literature for *E. coli* (Sigmund and Morgan, 1982; Vester and Garrett, 1987) and *Helicobacter pylori* (Versalovic et al., 1996).

[^{14}C]-phenylalanine incorporation in cell-free extracts from strains of intermediate resistance (<64 µg/ml), 306-2, 306-3, and 306-4 was as sensitive to azithromycin as extracts from the parent strain, 306-1 (Figure 4.6). This suggests that the mutation conferring resistance in these strains is not a mutation altering the antibiotic target (i.e. the ribosome), and may be membrane-associated. Further research is needed to determine the molecular basis of azithromycin resistance in these strains.

Table 4.1. Determination of optimal lysis conditions for preparation of *M. avium* S-20 extract. S-20 protein was recovered from 100 ml cultures as described in materials and methods.

Sample preparation	S-20 Protein (mg/ml)
*0.1 mm glass beads, 50s	0.35
*0.1 mm glass beads, 50s x 3	0.61
*0.5 mm glass beads, 50s	0.1
*0.5 mm glass beads, 50s x 3	0.48
**0.1 mm glass beads, 50s x 3	1.2
**0.1 mm glass beads, 50s x 5	1.3

*Data from parallel experiments with *M. avium* 306-3.

**Data from parallel experiments with *M. avium* 306-3.

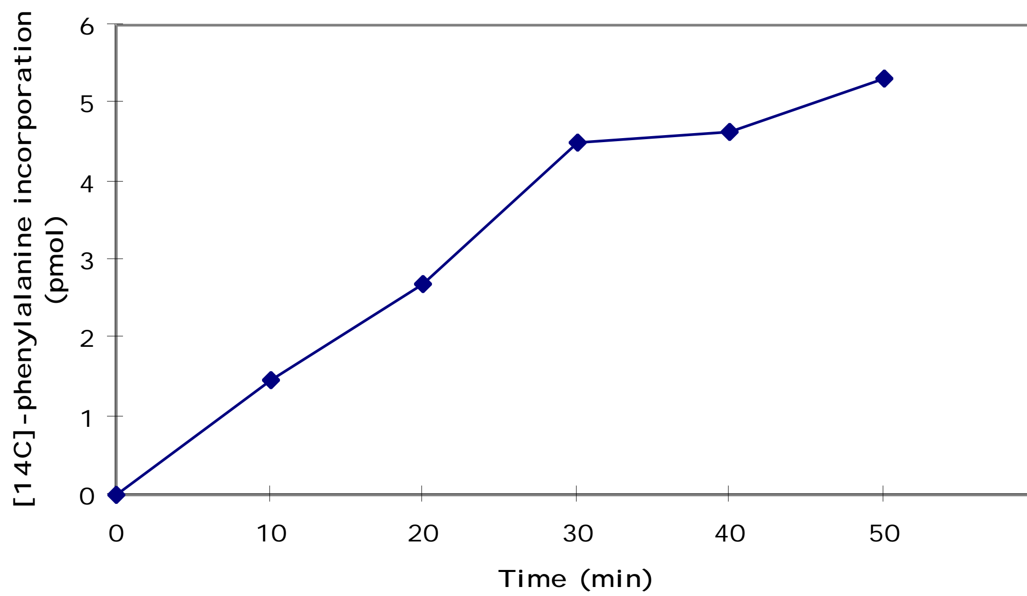


Figure 4.1. Kinetics of incorporation of [^{14}C]-phenylalanine into protein.

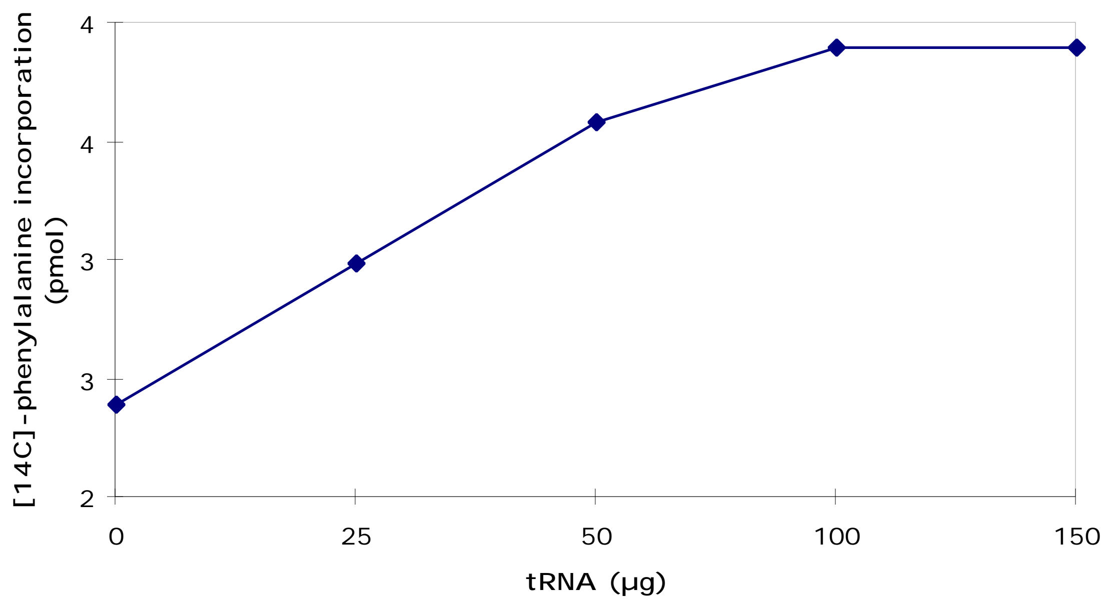
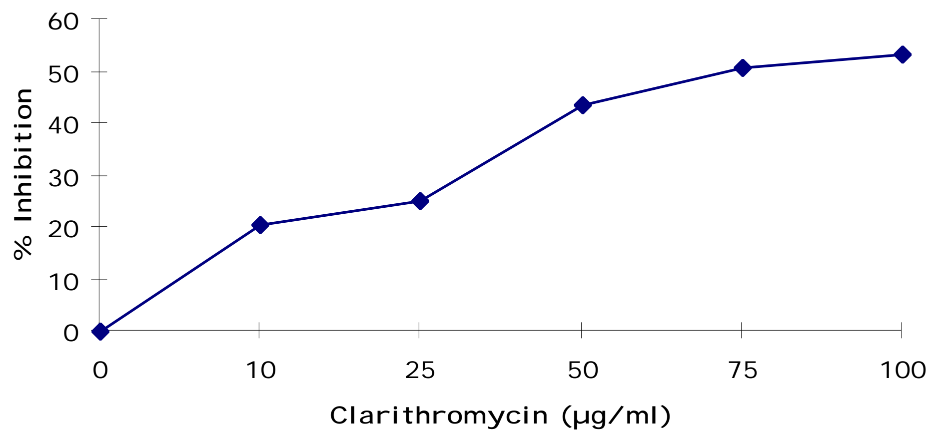
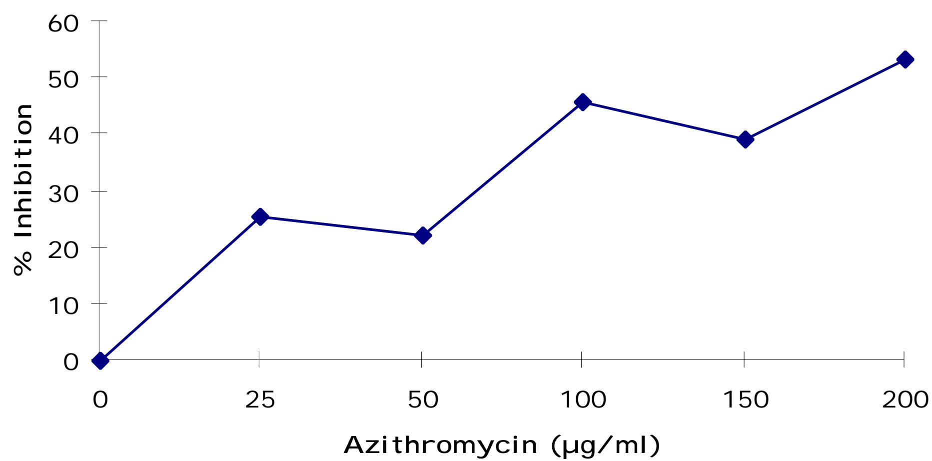


Figure 4.2. Incorporation of [^{14}C]-phenylalanine relative to tRNA concentration.



A.



B.

Figure 4.3. Effect of azithromycin and clarithromycin on [^{14}C]-phenylalanine incorporation in susceptible *M. avium* strains.

Panel A: *M. avium* 201-1 (CLA^S); Panel B: *M. avium* 306-1 (AZI^S)

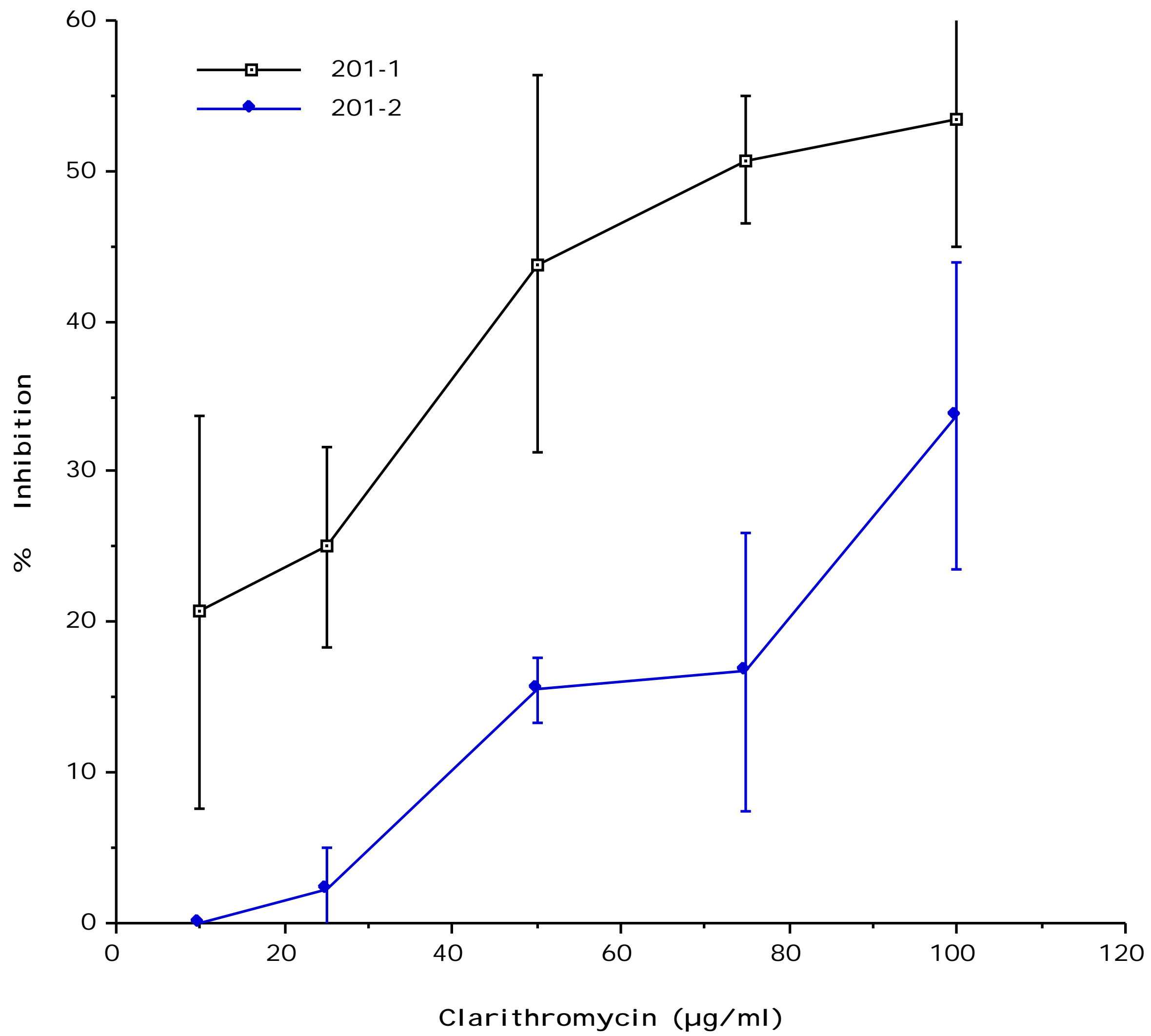


Figure 4.4. Effect of clarithromycin on [^{14}C]-phenylalanine incorporation in clarithromycin-sensitive and -resistant *M. avium* strains.

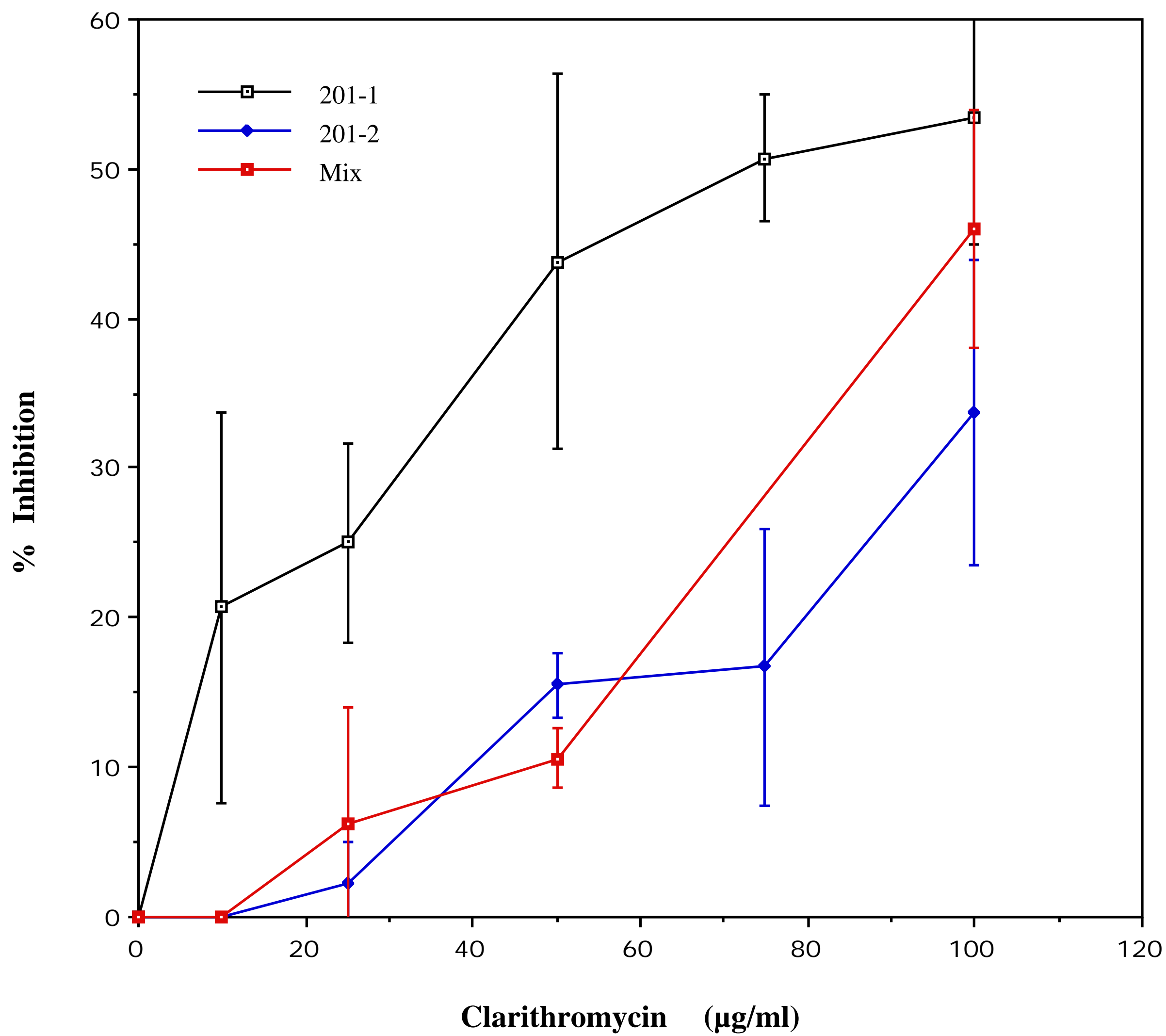


Figure 4.5. Effect of clarithromycin on [^{14}C]-phenylalanine incorporation in equal mixtures of S-20 protein from *M. avium* strains 201-1 (sensitive) and 201-2 (resistant).

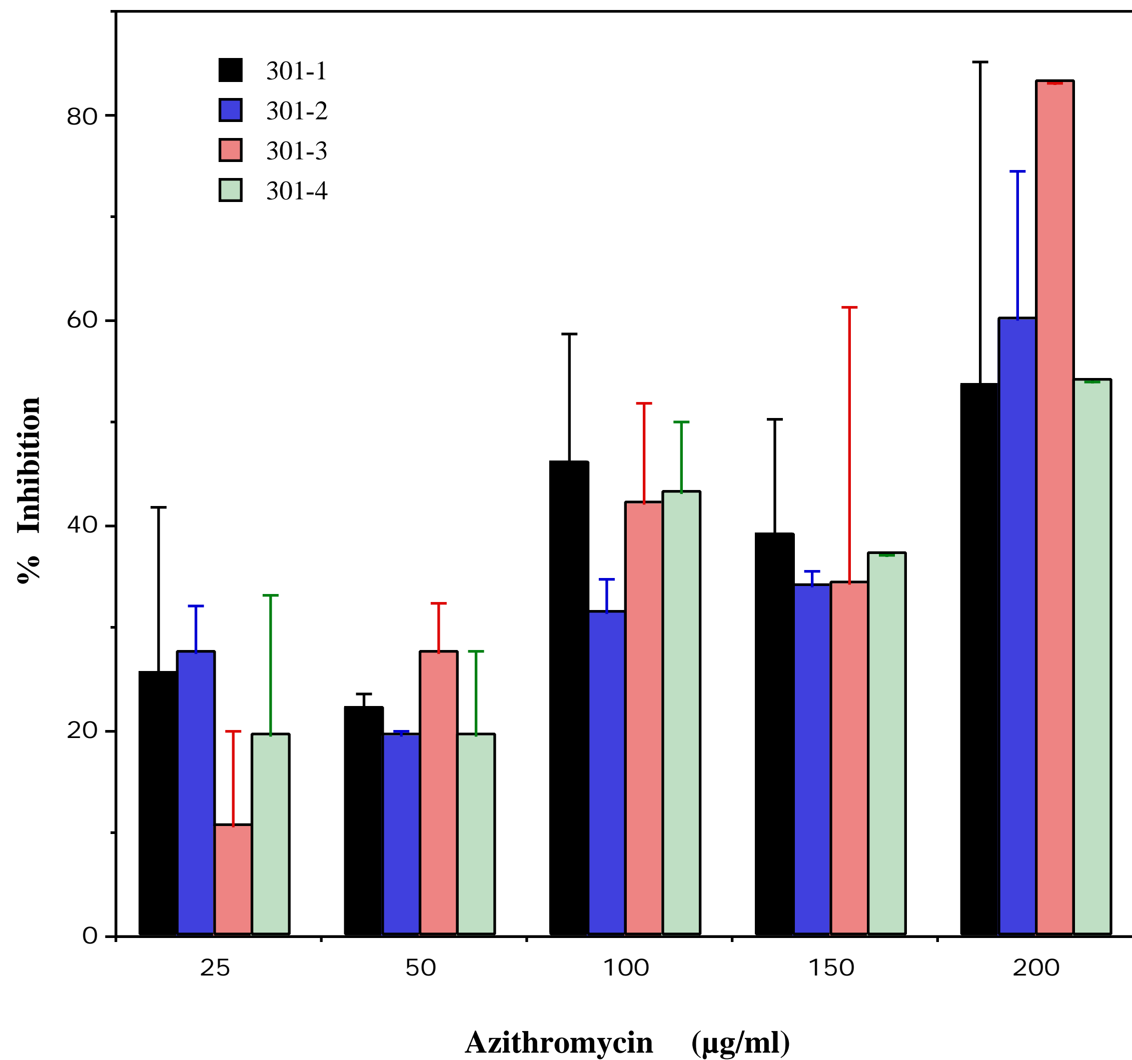


Figure 4.6. Effect of azithromycin on [^{14}C]-phenylalanine incorporation in *M. avium* strains exhibiting intermediate azithromycin-resistance.

Comparison of azithromycin and clarithromycin inhibition in the cell-free system and in intact cells. The MIC of clarithromycin for strain 201-1 was 0.8 μg/ml, and the MIC of azithromycin for strain 306-1 was 8 μg/ml. However, as illustrated in Figure 4.3, antibiotic concentrations for 50% inhibition of protein synthesis in the cell-free system were 45-50 μg/ml clarithromycin and 180-200 μg/ml azithromycin. This 50-fold difference in concentrations between the two systems may be due to the influence of two factors. First, it has been reported that high-pK macrolides are more potent at inhibiting growth than inhibiting protein synthesis in cell-free systems of *Staphylococcus aureus* (Mao and Weigand, 1968). Second, it has been reported that polyU-directed amino acid incorporating systems are more resistant to macrolide antibiotics than polyA- or polyC-directed systems (Wilhelm and Corcoran, 1967; Mao and Robishaw, 1971). Specifically, amino acid incorporation in a cell-free system from *Bacillus subtilis* (MIC = <1 μg/ml erythromycin) was maximally inhibited by 1.4 μg/ml erythromycin in a polyA-directed system, 7.0 μg/ml in a polyC-directed system, and 28 μg/ml in a polyU-directed system (Wilhelm and Corcoran, 1967). The reason polyU-directed incorporation is more resistant to macrolides than polyA- or polyC-directed incorporation is unknown.

Chapter 5

Characterization of *Mycobacterium avium* isolates exhibiting intermediate resistance to azithromycin.

ABSTRACT

The mechanism of intermediate resistance to azithromycin in *Mycobacterium avium* was investigated. Three isogenic *M. avium* strains exhibiting step-wise resistance to azithromycin were characterized in comparison to the sensitive parent. All strains were similar in hydrophobicity, growth medium requirements, and growth response to temperature, although growth of the parent strain was somewhat inhibited at 43˚C. The three azithromycin-resistant strains were resistant to a number of unrelated antibiotics and chemicals, including ciprofloxacin, rifabutin, and ethidium bromide. Addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an energy uncoupler, did not lower MICs for ciprofloxacin or ethidium bromide, indicating that the mechanism of resistance was not an energy-requiring process. Previous studies using cell-free extracts of the strains in an amino acid-incorporating system showed that they were as sensitive to azithromycin *in vitro* as the cell-free extract from the parent strain. The results rule out inactivation or efflux, and are not consistent with mutations in the target ribosome, although they are consistent with resistance due to altered permeability of the cell wall or cell membrane.

INTRODUCTION

Resistance to clarithromycin and azithromycin in clinical isolates of *Mycobacterium avium* has been a major focus of study over the past several years. Research has shown that *M. avium* strains resistant to high levels of azithromycin often have a mutation at position A-2058 or A-2059 in the single-copy 23S rRNA gene (Meier et al., 1994; Nash and Inderleid, 1995; Jensen-Cain and Falkinham, 1997). However, *M. avium* strains have been isolated that exhibit intermediate resistance to azithromycin and lack a mutation in the 23S rRNA gene. In *in vitro* poly U-dependent translation assays (Chapter 4), cell-free extracts from the intermediate resistant strains had similar sensitivity to azithromycin as the parent strain, suggesting that the mutation conferring resistance in these strains is not a mutation altering the antibiotic target (i.e. the ribosome). Potential mechanisms of resistance that do not involve modification of the target are antibiotic inactivation (Barthelemy et al., 1984; Kuo et al., 1989; Marshall et al., 1989), active efflux (Goldman and Capobiance, 1990; Ross et al., 1990), and permeability changes in the cell wall or cell membrane (Leclercq and Courvalin, 1991).

Inactivation of macrolide antibiotics has been reported in acid-fast relatives of the mycobacteria, namely *Nocardia* (Yazawa et al., 1994) and *Streptomyces* species (Jenkins and Cundiffe, 1991). Yazawa et al. (1994) describe five pathogenic species of *Nocardia* that inactivated erythromycin, midecamycin, and rokitamycin by glycosylation, reduction, deacylation, and/or phosphorylation of the antibiotic. Further, glycosylation has been demonstrated as a mechanism of macrolide resistance in streptomycetes (Kuo et al., 1989; Jenkins and Cundiffe, 1991).

Macrolide resistance mediated by active efflux has been reported in *Staphylococcus aureus* (Matsuoka et al., 1995) and *Staphylococcus epidermidis* (Goldman and Capobiance, 1990). Such active efflux systems are responsible for resistance to other antimicrobial agents such as fluoroquinolones, ethidium bromide, heavy metals, and detergents (Matsuoka et al., 1995; Takiff et al., 1996). Further, efflux pumps are inhibited by energy uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or 2,4-dinitrophenol (DNP), which disrupt the proton gradient (Liu et al., 1996). Recently, a strain of *Mycobacterium smegmatis* was described in which mutations affecting a chromosomal efflux gene, *lfrA*, conferred intermediate resistance to fluoroquinolones, ethidium bromide, tetracycline, and acriflavin (Takiff et al., 1996). Resistance of this strain to macrolide antibiotics was not tested. Mutations affecting *lfrA* did not alter the amino acid sequence, but appeared to increase expression of the encoded protein (Takiff et al., 1996). Hybridization studies have shown that a gene homologous to *lfrA* exists in *M. avium* (Takiff et al., 1996), suggesting the possibility that active efflux may mediate antibiotic resistance in this species.

In this report we describe the investigation of inactivation, efflux, and permeability changes as potential mechanisms of intermediate resistance to azithromycin in *M. avium*. The strains were characterized by measuring their hydrophobicity and determining growth medium requirements and growth response to temperature. Susceptibility to unrelated antibiotics and chemicals was also measured, as well as the effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on MICs of the strains.

MATERIALS AND METHODS

Bacterial strains. *M. avium* strains 306-1, 306-2, 306-3, and 306-4 are pre-treatment (306-1) and post-treatment (306-2, 306-3, and 306-4) isogenic isolates recovered at successive intervals from an HIV-infected patient with disseminated *M. avium* disease. The relatedness of the isolates was determined by DNA fingerprinting (Chapter 2). *M. avium* strain 201-1 and 201-2 are isogenic azithromycin-sensitive (201-1) and -resistant (201-2) strains used as controls in these experiments, and are described in further detail in Chapter 4.

Antibiotics and chemicals. Clarithromycin was donated by Abbott Laboratories (Chicago, IL), azithromycin was donated by Pfizer (Groton, CT), rifabutin was donated by Adria Laboratories (Dublin, OH), ciprofloxacin was donated by Miles (West Haven, CT) and clofazamine was donated by Ciba-Geigy (Sufferer, NY). Kanamycin sulfate, D-cycloserine, ethidium bromide, cadmium acetate, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and n-hexadecane were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions of clarithromycin, azithromycin, rifabutin, and clofazamine were prepared in dimethyl sulfoxide at a concentration of 20 mg/ml. Stock solutions of ciprofloxacin, kanamycin sulfate, ethidium bromide, and cadmium acetate were prepared in sterile distilled water at a concentration of 10 mg/ml. A stock solution of CCCP was prepared in ethanol at a concentration of 5 mg/ml.

Inactivation of azithromycin. Inactivation was assayed using a modification of the procedure of Yazawa et al. (1994). Each *M. avium* strain was inoculated into 5 ml Middlebrook 7H9 broth (BBL Microbiological Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) enrichment (BBL Microbiological Systems, Cockeysville, MD). Cultures were incubated for two days at 37˚C, and azithromycin was added to a final concentration of 30 µg/ml. Incubation continued for four more days at 37˚C, and the bacteria were harvested by centrifugation at 5,000 x g for 10 min. The resulting supernatant was filter-sterilized through a 0.2 µm filter, inoculated with one colony of *Mycobacterium smegmatis* VT307 (MIC = 3.75 µg/ml), and incubated for two days at 37˚C. Each tube was observed for growth of *M. smegmatis* as an indicator of azithromycin inactivation.

Temperature sensitivity and media requirements. *M. avium* strains 306-1, 306-2, 306-3, and 306-4 were inoculated into 5 ml Middlebrook 7H9 broth containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OADC enrichment, and incubated at 37˚C for 7 days. 0.01 ml of the culture was mixed with 5 ml tempered Middlebrook 7H9 broth containing 0.7% agar, and poured onto Middlebrook 7H10 (BBL Microbiological Systems, Cockeysville, MD) agar plates containing 0.5% (vol/vol) glycerol and either 10% (vol/vol) OADC enrichment, 0.5% (vol/vol) Tween 80 and 10% (vol/vol) albumin-dextrose-catalase (ADC) enrichment (Difco Laboratories, Detroit, MI), or 10% (vol/vol) ADC enrichment. Each strain, in duplicate, was incubated at the following temperatures: 21˚C, 30˚C, 37˚C, and 43˚C. In addition, each strain was subjected to heat shock at 55˚C for 1.5 hours, then plated on the three types of media and incubated at 37˚C. Plates were initially examined in 5 days, and incubated for a total of 28 days. Growth was defined as the appearance of single, isolated colonies on the agar, not within the primary streak.

Cross-resistance to other antibiotics and chemicals. Middlebrook 7H10 agar plates were prepared containing 0.5% glycerol (vol/vol), 10% (vol/vol) OADC enrichment, and antibiotics or chemicals at appropriate concentrations. Antibiotics used were azithromycin, clarithromycin, ciprofloxacin, clofazamine, D-cycloserine, kanamycin, and rifabutin. Chemicals used were ethidium bromide, cadmium acetate, and CCCP. CCCP was also used in combination with other agents to assess the effect of energy uncouplers on susceptibility, and thus assess the

likelihood of efflux as a potential mechanism of resistance. *M. avium* strains 306-1, 306-2, 306-3, and 306-4 were streaked onto plates using sterile disposable loops, and incubated at 37˚C for 14 days. Plates containing ethidium bromide were incubated in the dark at 37˚C. Resistance was defined as the appearance of single, isolated colonies on the agar after 14 days.

Measurement of hydrophobicity. Adherence of *M. avium* strains to n-hexadecane was measured using the following procedure, modified from Stormer and Falkinham (1989). Each *M. avium* strain was inoculated into 5 ml Middlebrook 7H9 broth containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OADC enrichment (BBL Microbiological Systems, Cockeysville, MD). Cultures were incubated for five to seven days at 37˚C, then harvested by centrifugation for 10 min at 5,000 x g. After washing twice in phosphate buffered saline (PBS; 1 mM NaH_2PO_4, 7.5 mM NaCl, pH 7.2), the cell pellet was resuspended in PBS, and adjusted to an optical density of 0.3 at 580 nm with PBS. One ml of the adjusted suspension was transferred to a microfuge tube, and 0.066 ml n-hexadecane was added. The samples were vortexed 2 min, then allowed to stand at room temperature for 20 min. The aqueous (lower) layer was removed, transferred to a glass cuvette, and the optical density at 580 nm was determined. Hydrophobicity was expressed as the percent adherence to n-hexadecane, indicated by a decrease in optical density as cells partitioned into the n-hexadecane layer.

RESULTS AND DISCUSSION

Inactivation of azithromycin. Growth of *M. smegmatis* was inhibited in the filter-sterilized supernatant from the *M. avium* cultures containing 30 µg/ml azithromycin (Table 4.1). However, the *M. smegmatis* strain did grow in the filter-sterilized supernatant from *M. avium* 306-1 that did not contain azithromycin, indicating that inhibition of *M. smegmatis* growth in the antibiotic-containing samples was due to the action of azithromycin and not to depletion of nutrients in the medium. The data from this bioassay indicated that *M. avium* strains 306-2, 306-3, and 306-4 were not inactivating azithromycin. This is not unexpected, as the organism would have to acquire the genes encoding inactivation enzymes (Barthelemy et al., 1984), and resistance conferred by lateral transfer of plasmids has never been found as a mechanism of resistance in slowly growing mycobacteria (Jensen and Falkinham, 1996).

Temperature sensitivity and media requirements. All four strains exhibited growth at 21˚C, 30˚C, 37˚C, and 43˚C, as well as at 37˚C following heat shock at 55˚C on M7H10 plates supplemented with either 10% OADC or 0.5% Tween 80 plus 10% ADC. None of the strains were able to grow on M7H10 containing only ADC, which reflects the requirement of slowly-growing mycobacteria for oleic acid (Kajioka and Hui, 1978). Colonies appeared on plates incubated at 30˚C, 37˚C, and 43˚C by the tenth day of incubation, whereas colonies did not appear on plates incubated at 21˚C until after 20 days. Although all the strains exhibited growth across the temperature range, 306-1 growth was impaired at 43˚C compared with the other strains, suggesting this strain may be slightly temperature-sensitive. It is unknown whether there is a correlation between temperature sensitivity and the antibiotic susceptibility of 306-1.

Cross-resistance to other antibiotics and chemicals. *M. avium* strains 306-1, 306-2, 306-3, and 306-4 were all resistant to >50 µg/ml kanamycin sulfate, >50 µg/ml D-cycloserine, and >0.4 mM cadmium acetate. All four strains were sensitive to <1 µg/ml clofazamine. Susceptibilities for the remaining antibiotics and chemicals tested varied among the strains, and are presented in Table 4.3. As a general trend, strains 306-2, 306-3 and 306-4 exhibited increasing resistance to the agents tested compared with 306-1. Strain 306-4, in particular, was highly resistant to ethidium bromide (64 µg/ml) and ciprofloxacin (16 µg/ml) compared with the parent strain, 306-1. Addition of 15 µg/ml or 25 µg/ml CCCP had little effect on the MICs of the agents tested, as MICs only showed a 0- to 1-fold decrease (Table 4.4). In contrast, Takiff et al. reported that CCCP reduced the MICs 2- to 4-fold of drugs to which resistance was conferred by active efflux in *M. smegmatis*. These results indicate that cross-resistance in *M. avium* strains 306-2, 306-3, and 306-4 was not due to an energy-requiring process such as efflux. Interestingly, strain 306-4 was more resistant to CCCP alone (60 µg/ml) than the other three strains (45 µg/ml), suggesting that altered permeability may be a factor in resistance in strain 306-4.

Changes in hydrophobicity. As illustrated in Figure 4.1, *M. avium* strains 306-1, 306-2, 306-3, and 306-4 exhibited no significant differences in their adherence to n-hexadecane, indicating no major alterations in cell-surface hydrophobicity. However, the 306 strains are much more hydrophobic than strains 201-1 and 201-2.

Mechanism of intermediate resistance to azithromycin. The data presented in this report provide strong evidence that intermediate resistance to azithromycin in strains 306-2, 306-3, and 306-4 is not due to antibiotic inactivation or active efflux. In addition, results with the energy uncoupler CCCP suggest that no energy-requiring processes are involved in resistance. Further, resistance did not result from mutations in the peptidyl transfer region of the 23S rRNA gene (Chapter 3), and cell-free extracts from strains 306-2, 306-3, and 306-4 exhibited similar

Table 5.1. Inactivation of azithromycin by *M. avium* strains.

M. avium strain	AZI conc. (μg/ml)	Growth of *M. smegmatis*
306-1	0	+
306-1	30	-
306-2	30	-
306-3	30	-
306-4	30	-

Table 5.2. Temperature sensitivity and growth requirements of *M. avium* strains.

	30˚C			37˚C			43˚C			50˚C, then 37˚C		
Strain	OADC	ADC+T	ADC	OADC	ADC+T	ADC	OADC	ADC+T	ADC	OADC	ADC+T	ADC
306-1	++	+	-	++	+	-	+	±	-	++	+	-
306-2	++	+	-	++	+	-	++	+	-	++	+	-
306-3	++	+	-	++	+	-	++	+	-	++	+	-
306-4	++	+	-	++	+	-	++	+	-	++	+	-

(Plates were incubated for 14 days.)
++ represents colony sizes > 1.5 mm
+ represents colony sizes 1.5 mm
± represents colony sizes < 1mm
- represents no growth

Abbreviations: OADC, 10% oleic acid-albumin-dextrose-catalase enrichment; ADC+T, 10% albumin-dextrose-catalase enrichment plus 0.5% Tween 80; ADC, 10% albumin-dextrose-catalase enrichment.

Table 5.3. Minimal inhibitory concentrations (MICs) of *M. avium* isolates.

M. avium strain	Minimal Inhibitory Concentration (μg/ml)				
	AZI	CIP	CLA	EtBr	RFB
306-1	>10	4	2	8	1
306-2	40	8	2	8	1
306-3	40	4	4	32	5
306-4	60	16	5	64	5

Abbreviations: AZI, azithromycin; CIP, ciprofloxacin; CLA, clarithromycin; EtBr, ethidium bromide; and RFB, rifabutin.

Table 5.4. Effect of CCCP on MICs of *M. avium* isolates.

M. avium strain	Minimal Inhibitory Concentration (µg/ml)						
	CCCP	CIP	CIP + CCCP*	CIP + CCCP**	EtBr	EtBr + CCCP*	EtBr + CCCP**
306-1	45	4	2	2	8	4	<4
306-2	45	8	8	2	8	8	<4
306-3	45	4	2	2	32	32	32
306-4	60	16	16	>8	64	>32	>32

*Concentration of CCCP was 15 µg/ml.
**Concentration of CCCP was 25 µg/ml.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CIP, ciprofloxacin; EtBr, ethidium bromide

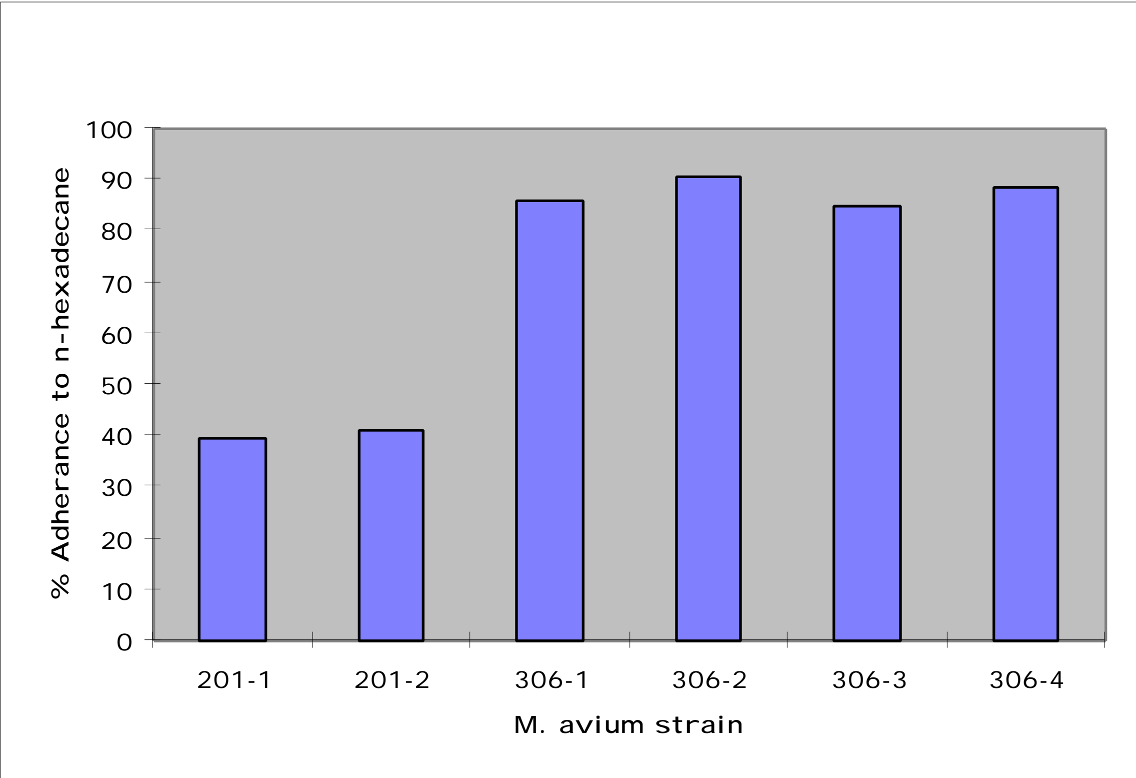


Figure 4.1. Hydrophobicity of *M. avium* isolates.

sensitivity to azithromycin as the parent strain, 306-1 in polyU-directed *in vitro* translation assays (Chapter 4), suggesting that modification of the antibiotic target is not a mechanism of azithromycin resistance. The increasing, step-wise resistance of the isolates recovered sequentially from a single parent also suggests that multiple mutations may be involved in resistance in these strains. Further, although the strains did not vary in hydrophobicity, the cross-resistance of strains 306-2, 306-3, and particularly 306-4 to other, unrelated antibiotics or chemicals strongly suggests that altered permeability of the cell wall or cell membrane is a mechanism of intermediate resistance in these *M. avium* strains. Further studies will be required to identify the gene or genes involved in intermediate resistance to azithromycin in *M. avium.*

Appendix A:

Cloning and isolation of ribosomal proteins from *Mycobacterium* species.

ABSTRACT

Genes encoding putative ribosomal proteins L4 and L22 were amplified from *Mycobacterium smegmatis* VT307 by PCR using degenerate primers and cloned into pBluescriptSK+. Sequence analysis suggests that the mycobacterial homologue of L22 may have been isolated using this method, based on the similarity of the hydropathy plot of the deduced amino acid sequence to hydropathy plots of other proteins in the database. Further, a method was developed for isolating ribosomal proteins from *Mycobacterium avium* that may provide a useful tool for investigating mechanisms of high-level macrolide resistance that do not involve mutations in 23S rRNA by allowing analysis of ribosomal proteins from sensitive and resistant strains.

INTRODUCTION

Macrolide antibiotics are bacteriostatic agents that inhibit protein synthesis by binding to a common region on the 50S ribosomal subunit and preventing translocation (Pestka, 1977). The binding site involves domain V of the 23S rRNA and several ribosomal proteins, including L4, L15, L16, and L22 (Pestka, 1977).

Due to the similarity among macrolide antibiotics, studies of macrolide-resistance in other bacteria can be used to guide studies of *M. avium*. Although the majority of high-level (>64 μg/ml) macrolide-resistant *M. avium* isolates possess mutations at positions 2058 or 2059 in domain V of the 23S rRNA, approximately 10% of high-level resistant strains do not have such mutations (Meier et al., 1994; Nash and Inderleid, 1995) . Thus, high-level resistance must be due to an alternate mechanism in those strains. In *E. coli,* erythromycin resistance has been shown to be due to mutations in ribosomal proteins L4 or L22 that altered the electrophoretic mobility of these proteins in two-dimensional polyacrylamide gel electrophoresis (Otaka et al., 1970; Wittman et al., 1973). Because of the conservation of ribosomal proteins in bacteria and the apparent limited sites for mutations in ribosomal proteins leading to macrolide-resistance (Wittman et al., 1973), it is possible that *M. avium* mutations will occur at the same sites. The objective of the following experiments was to determine if alterations in ribosomal proteins lead to macrolide-resistance in *M. avium*. The approach involved cloning and sequencing the genes encoding mycobacterial L4 and L22 ribosomal proteins, and isolation and analysis of *M. avium* ribosomes and ribosomal proteins.

MATERIALS AND METHODS

Bacterial strains. *Mycobacterium avium* strains used in this study were 306-1, 306-2, 306-3, 306-4, and 201-1. The 306 series of strains were isolated from an AIDS patient treated with azithromycin and each isolate exhibits increasing resistance to azithromycin. Strain 201-1 is a clinical isolate that is macrolide-sensitive. The *Mycobacterium smegmatis* strain used in this study was VT307.

Primer design and PCR amplification. Oligonucleotide primers were designed to amplify mycobacterial homologues of the genes encoding ribosomal proteins L4 and L22. Amino acid sequences of L4 and L22 from other bacterial species were aligned using the MegAlign module in DNAStar, and conserved regions among the genes were identified (Figures A.1 and A.2). Degenerate primers were designed based on the deduced DNA sequences in these conserved regions (Table A.1). Touchdown PCR reactions to amplify the mycobacterial homologues contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.7 mM $MgCl_2$, 0.3 mM each deoxyribonucleotide, 30 pmol of each oligonucleotide primer, 1 unit *Taq* DNA polymerase, and 10 ng of *M. smegmatis* DNA. Reactions were subjected to an initial denaturation at 95˚C, followed by 30 cycles of 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 2 min, with the annealing temperature ramped from 55˚C to 40˚C during the 30 cycles. This was followed by 10 cycles of 94˚C for 1 min, 40˚C for 1 min, and 72˚C for 2 min. Products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Cloning and sequencing of PCR products. Amplification products from *M. smegmatis* using each set of primers were excised from a low melting point agarose gel and cloned into pBluescript SK+ digested with *Sma*I and T-tailed, using the TA cloning method (Ausubel et al, 1995). Single-stranded DNA was generated from each clone using VCSM13 helper phage (Stratagene, San Diego, CA) and was sequenced using Sequenase 2.0 according to manufacturers instructions (U.S. Biochemical, Cleveland, OH). The sequences were analyzed using DNAStar (Madison, WI).

Southern blotting. The *rplD* and *rplV* genes were amplified from *Escherichia coli* DNA using the PCR methods described in Chapter 3. Primer sequences are listed in Table A.1. Unincorporated dNTPs and primers were removed using a Magic PCR column (Promega, Madison, WI) and the products were biotin-labeled via random priming using the Images® system (U.S. Biochemical Co., Cleveland, OH). Clones containing mycobacterial DNA were digested with *Bam*HI and *Eco*RI, and electrophoresed on a 0.8% agarose gel. The DNA fragments were depurinated in 0.3N HCl, denatured in 0.4N NaOH, and the DNA transferred to a nylon membrane by Southern blotting overnight in 0.4 N NaOH. Following prehybridization for 2 hours at 45˚C, the appropriate probe was added to a final concentration of 10 ng/ml, and hybridization continued overnight at 45˚C. Post-hybridization washes and detection of bound probe were performed according to the manufacturer's instructions (U.S. Biochemical, Cleveland, OH).

Sucrose-density gradient ribosome isolation. *M. avium* strains 306-1, 306-2, 306-3, and 306-4 were cultured in 40 ml Middlebrook 7H9 broth containing 10% OADC (vol/vol) and 0.5% (vol/vol) glycerol, and incubated 5-7 days at 37˚C. Cells were harvested by centrifugation (8,000 x g for 10 min at 4˚C) and washed twice in cold standard buffer, pH 7.8, containing 50 mM Tris, 10 mM magnesium acetate, 60 mM ammonium chloride, and 6 mM ß-mercaptoethanol before resuspending in 1 ml of the same buffer (Yamada et al., 1972). The cell suspension was

```
     M - E L V L K D A X G A - - - E L T V S D T V F G I D F N E A L V H Q V V V A Q  Majority
                       10                  20                  30                  40
1    M P K V A L Y N Q N G Q T V G E I E L N D A V F G I E P N K H V L F E A V I M Q  Bacillus
1    M - E L V L K D A Q S A - - - - L T V S E T T F G R D F N E A L V H Q V V V A Y  Escherichia
1    M - K L Q V L D T K G N E I K E I A L N D Y V W G I E P H Q Q A I Y D T V I S Q  Mycoplasma
1    M - E L V M K D A P G A - - - - L T V S E T T F G R D F N E A L V H Q V V V A Y  Yersinia

     A A G A R Q G T R A Q K T R A E V S G G G K K P W R Q K G T G R A R Q G S I K A  Majority
                       50                  60                  70                  80
41   R A S M R Q G T H K T[K N R A E V S G]G G R K P W R Q K G T G R A R Q G S I R A  Bacillus
36   A A G A R Q G T R A Q[K T R A E V T G]S G K K P W R Q K G T G R A R S G S I K S  Escherichia
40   Q A A L R Q G T K K V[K T R A E V S G]G G R K P . K Q K G T G L A R Q G S I R A  Mycoplasma
36   A A G A R Q G T R A Q[K T R A E V T G]S G K K P W R Q K G T G R A R A G S V K S  Yersinia

     P I W R G G G V T F G A X P Q - D H S Q K V N K K V R R G A L K S I L S E L V R  Majority
                       90                  100                 110                 120
81   P Q W R G G G T V F G P V P R - S Y S Y K L P K K V R R L A I K S A L S S K V L  Bacillus
76   P I W R S G G V T F A A R P Q - D H S Q K V N K K M Y R G A L K S I L S E L V R  Escherichia
80   P Q . K G G E V T F G P T P D I N Y K K S V N K K V R A L A F R S V L S L K V K  Mycoplasma
76   P I W R S G G V T F A A K P Q - D H S Q K V N K K M Y R G A L K S I L S E L V R  Yersinia

     E D R L V V V D K F S V E A P K T K L L A Q K L K D L A L D - D K V L I V T G E  Majority
                       130                 140                 150                 160
120  E N D I V V L D Q L S L E A P K T K E M V K I L N N L S V D - R K A L I V T D E  Bacillus
115  Q D R L I V V E K F S V E A P K T K L L A Q K L K D M A L E - D - V L I I T G E  Escherichia
120  E N N L V I V D K F D F A K P S T K E M V V V M K N L K I D D Q K T L I V T K E  Mycoplasma
115  Q D R L I I V E K F S V E A P K T K L L A Q K L K D M A L E - D - V L I V T G E  Yersinia

     L D E N V F L A A R N L X G V D V R D A N G I D V V S L L A F D K V V I T A D A  Majority
                       170                 180                 190                 200
159  L N E N V Y L S A R N I P G V K V V P A N G I N V L D V L N H D K L V I T K A A  Bacillus
153  L D E N L F L A A R N L H K V D V R D A T G I D P V S L I A F D K V V M T A D A  Escherichia
160  K E E L V V K S S N N I T G V K T I S A N Q L N V F D L L N A T K L L I T E E A  Mycoplasma
153  L D E N L F L A A R N L Y K V D V R D V A G I D P V S L I A F D K V V M T A D A  Yersinia

     V K Q V E E V L A -                                                              Majority
                       210
199  V E K[V E E V L A].                                                              Bacillus
193  V K Q[V E E M L A].                                                              Escherichia
200  A I A[V E E V Y A].                                                              Mycoplasma
193  V K Q[V E E M L A].                                                              Yersinia
```

Figure A.1. Conserved regions in amino acid sequences of L4 ribosomal proteins from bacteria.

```
     M E A K A K X R T I R I A P R K V R L V A D L I R G K X V S  Majority
                       10                  20                  30

1    M E A K A I G K T I R I A P R K V R L V V D L I R G K N V K  acholeplasma translation
1    M Q A K A V A R T V R I A P R K A R L V I D L I R G K E V G  bacillus translation
1    M E T I A K H R H A R S S A Q K V R L V A D L I R G K K V S  ecoli translation
1    M E A K A K L S M I R I S P R K M R L V A D T I R N K A V S  Mycoplasma translation

     E A X A I L X X T N K X A A P X I X K V L X S A V A N A E H  Majority
                       40                  50                  60

31   E A Q A I L M F T P R G A S P V I A K V L D S A I A N R T H  acholeplasma translation
31   E R F A I L R H T P K A A S P I I E K V L K S A V A N A E H  bacillus translation
31   Q A L D I L T Y T N K K A A V L V K K V L E S A I A N A E H  ecoli translation
31   V A V A T L K N L N K D A A E P I L K L L N S A V A N A V N  Mycoplasma translation

     N X G M D X D N L X V K X I F V D E G P T L K R F R P R A K  Majority
                       70                  80                  90

61   N L N L N L E N L F V K E V W A N E S I T M K R M L P R A K  acholeplasma translation
61   N Y D M D V N N L V I S Q A Y V D E G P T L K R F R P R A M  bacillus translation
61   N D G A D I D D L K V T K I F V D E G P S M K R I M P R A K  ecoli translation
61   N N G M E A D K L Y V K T I F V N E G P T L K R F R P R A H  Mycoplasma translation

     G R A X X I X K R T S H I T V V V S D X K - - -              Majority
                      100                 110

91   G S G H L I R K R T S H I T V V V A E R E - - - .            acholeplasma translation
91   G R A S A I N K R T S H I T I V V S E K K E G .              bacillus translation
91   G R A D R I L K R T S H I T V V V S D - R - - - .            ecoli translation
91   G R A Y E I F K R T S H V V I V V S D E K - - - .            Mycoplasma translation
```

Figure A.2. Conserved regions in amino acid sequences of L22 ribosomal proteins from bacteria.

Table A.1. Sequences of oligonucleotide primers used in this study.

Primer	Sequence
EC-rplD-1	5' C C G T G C T G A A G T A A C T G G T 3'
EC-rplD-2	5' A T G C C A G C A C C T C C T C A A 3'
EC-rplV-1	5' C C T G A T T C G C G G T A A G 3'
EC-rplV-2	5' G T A A T G T G G C T T G T G C G T 3'
UrplV-1	5' G A Y Y T N A T H M G N G G N A A R 3'
UrplV-2	5' D A T R T G N Y R N G T N C K Y T T 3'
UrplD-1	5' A A R C C N T G G C G N G A R G G N A C N G G 3'
UrplD-2	5' T C A N G C N A G C A T Y T G Y T G N A C 3'

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; K, guanine and thymine; R, adenine and guanine; Y, cytosine and thymine; D, adenine, guanine, and thymine, N, adenine, cytosine, guanine, and thymine.

transferred to a tube containing 1 g chilled 0.1 mm glass beads, and cells were disrupted in a Mini-Bead Beater® (Biospec Products, Bartelsville, OK) by three cycles of 50 s agitation followed by 60 s on ice between each interval. The resulting lysate was centrifuged briefly (8,000 x g for 5 min at 4˚C) to pellet beads and cell debris, and the supernatant centrifuged for 20 min (20,000 x g at 4˚C). The supernatant was decanted and centrifuged an additional 20 min (20,000 x g at 4˚C). Following the second centrifugation, the supernatant was centrifuged for a final 10 min (20,000 x g at 4˚C). The ribosomes were isolated from the supernatant by differential centrifugation (100,000 x g at 4˚C) in a 50Ti fixed angle rotor in a Beckman L5-50 ultracentrifuge for 120 min. The ribosome-containing pellet was resuspended in 100 µl cold standard buffer, and dialyzed against standard buffer containing 0.1 mM $MgCl_2$ for 18 h at 4˚C. The sample was layered onto a 10% to 30% linear sucrose gradient in standard buffer, 0.1 mM $MgCl_2$, and centrifuged in cellulose acetate tubes in an SW27 rotor at 21,000 rpm for 18 hours at 4˚C. Half ml fractions were collected and the absorbance at 260 nm of each fraction was measured.

Northern dot blot. Samples from the sucrose gradient were mixed 1:1 with RNA dilution buffer (5:3:2 distilled water:20X SSC: formaldehyde), and 3 µl was spotted onto a nylon membrane and cross-linked to the membrane with UV-irradiation (Stratagene, La Jolla, CA). The membrane was pre-hybridized in high SDS buffer at 55˚C for 3 hours, then hybridized overnight at 55˚C with 10 ng/ml digoxigenin-conjugated 23S rRNA probe. Post-hybridization washes and detection were performed as described above.

RESULTS and DISCUSSION

Cloning and sequencing of a putative mycobacterial L22 homologue. A pool of products ranging in size from 250 to 300 bp were amplified from *M. smegmatis* VT307 DNA using primers UrplV-1 and UrplV-2, and subsequently cloned into pBluescript SK+. Hybridization of the transferred clones under non-stringent conditions (i.e. 45˚C hybridization temperature) with the heterologous *E. coli* probe led to identification of clones containing a 250 bp fragment. The plasmid was designated pML22. Sequence data generated from pML22 was compiled using the SeqEd and SeqMan modules of DNAStar. The resulting sequence is depicted in Figure A.3. Alignment of the amino acid sequence of pML22 with L22 protein sequences from *Escherichia coli, Bacillus stearothermophilus, Acholeplasma* and *Mycoplasma* (using the MegAlign module of LaserGene) showed regions of weak homology with the known L22 proteins (Figure A.4). In addition, a hydropathy plot (Kyte and Doolittle, 1982) of the ML22 protein shows a similar profile to that of the L22 proteins from the other bacteria (Figure A.5). The hydrophilicity results suggest that the mycobacterial DNA cloned into pML22 could be a portion of the mycobacterial homologue to ribosomal protein L22. However, the lack of substantial homology between the protein sequences of this clone and known ribosomal proteins makes this a tenuous conclusion. Attempts to clone and sequence the mycobacterial L4 homologue resulted in cloning the L4 gene amplified from an *E. coli* contaminant present in the PCR reaction.

Sucrose-density gradient ribosome isolation. Fractions 5-7 from the sucrose-density gradient were found to have a peak absorbance at 260 nm, suggesting these fractions contain rRNA and thus ribosomes (Figure A.6). Hybridization between RNA transferred from the gradient with a 23S rRNA gene probe from *M. avium* 201-1 confirmed that fractions 5-7 did contain ribosomal rRNA (Figure A.7).

```
TGATGTGGCAGGTGCGCTTCGGTGAACGACGTCAGTGACAATGTTGGTGC
™£££¢££££ ££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££  5
ACTACACCGTCCACGCGAAGCCACTTGCTGCAGTCACTGTTACAACCACG

CCGTGCTGCCGAACCCGTGCTGCGACAGGCTGTCGACGATGGGGAAGCCC
££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££  1
GGCACGACGGCTTGGGCACGACGCTGTCCGACAGCTGCTACCCCTTCGGG

CGGTCCTTGAGTTCGGCGGCGCTGGCGTGACCACCGTGGCGTCGATCTGG
££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££  1
GCCAGGAACTCAAGCCGCCGCGACCGCACTGGTGGCACCGCAGCTAGACC

CCCGAACTCAGACCCGCGATGGACTCCGGTGTCGGGACGTCGCGTACCTC
££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££  2
GGGCTTGAGTCTGGGCGCTACCTGAGGCCACAGCCCTGCAGCGCATGGAG

GATCTTCCCTCGGATCAGATCAGGGCTGCAGGAATTCGATATCWAGCTTA
££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££ ££££¢££££  2
CTAGAAGGGAGCCTAGTCTAGTCCCGACGTCCTTAAGCTATAGWTCGAAT

TCGATACCGTCGACCTC
££££¢££££ ££££¢£  267
AGCTATGGCAGCTGGAG
```

Figure A.3. DNA sequence of ML22, a putative *M. smegmatis* L22 gene.

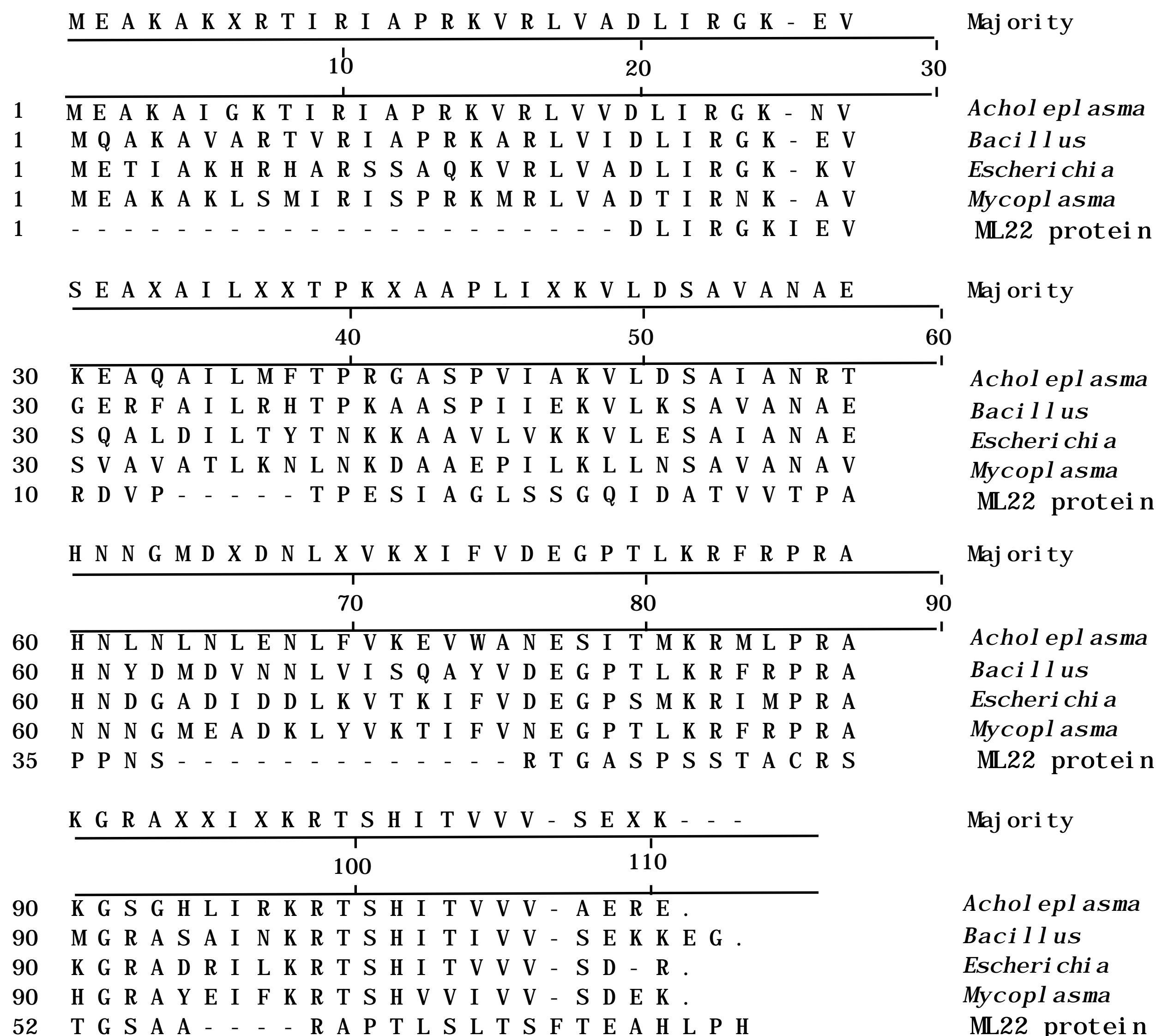


Figure A.4. Comparison of the ML22 deduced amino acid sequence with amino acid sequences from L22 ribosomal proteins in other bacteria.

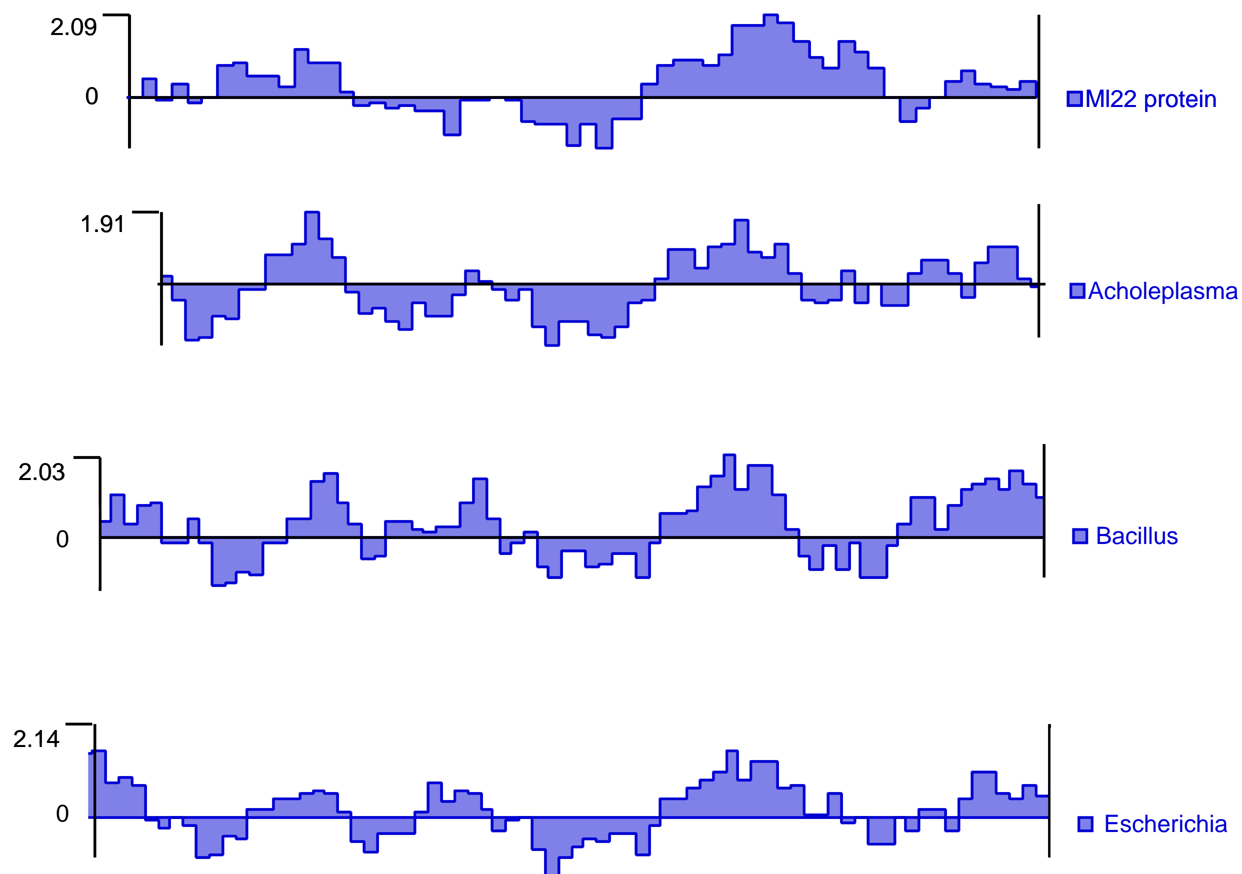


Figure A.5. Comparison of hydropathy plots of ML22 and known L22 ribosomal proteins.

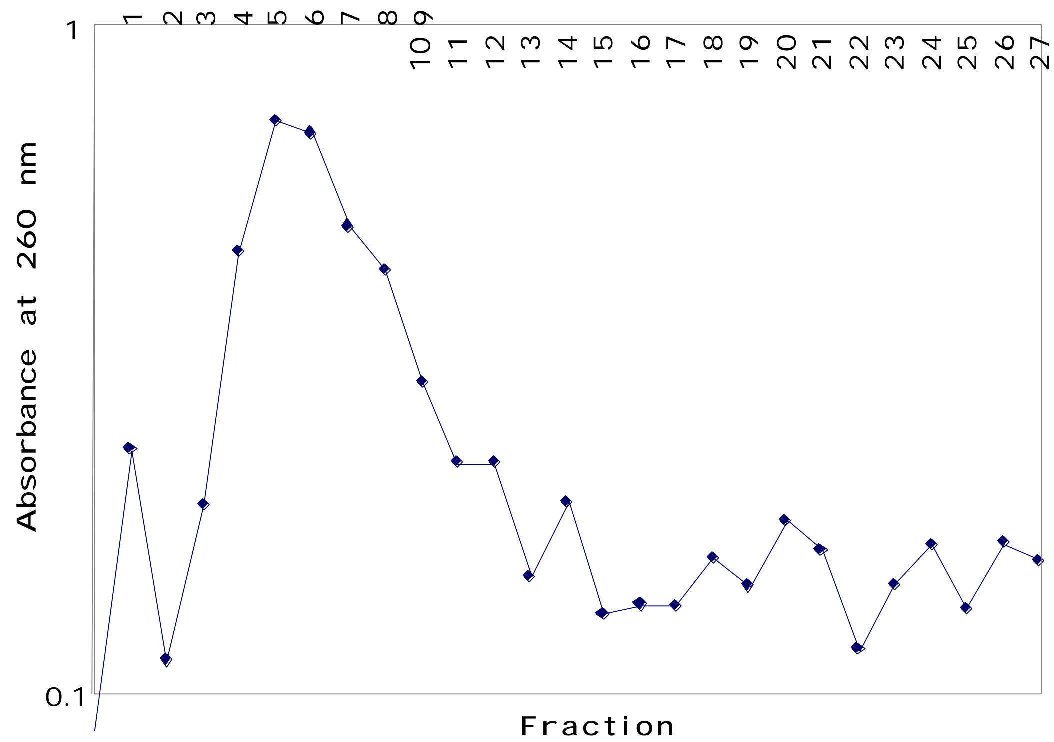


Figure A.6. Absorbance at 260 nm of fractions collected following sucrose-density gradient centrifugation.

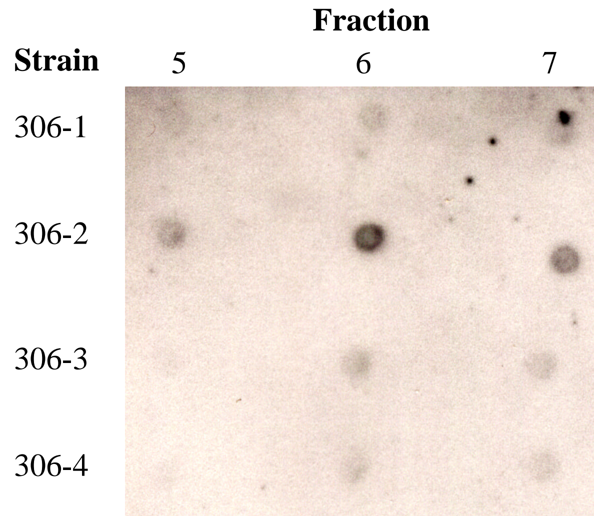


Figure A.7. Northern dot blot analysis of fractions collected following sucrose-density gradient centrifugation to isolate ribosomes. Fractions from *M. avium* strains were probed with a 23S rRNA gene as described in the materials and methods.

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Curriculum Vitae

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1992 B.S. in Biology, Magna Cum Laude, Southern College, Collegedale, TN.

Professional Experience

1992 - 1996 Graduate Teaching Assistant: Biology Department, VPI & SU, Blacksburg, VA. Genetics, Molecular Biology Laboratory, and Principles of Biology Laboratory.

1993 - Present Graduate Research Assistant: Biology Department, VPI & SU, Blacksburg, VA. Laboratory of Joseph O. Falkinham III, Ph.D. (Summers 1993-1996; Fall 1996; Spring 1997)

1996-Present Technical Consultant: Dominion Biosciences, Inc., Corporate Research Center, Blacksburg, VA.

Cumulative Teaching Evaluations 3.5 to 4.0 (on 4.0 scale)

Honors and Awards

1996 Virginia Tech Outstanding Graduate Teaching Assistant Award (university-wide)
1995 - 1996 State Tuition Scholarship
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Grants and Fellowships

Arnold Ravin-Murial Rogers Fellowship. June 1994.

Grant "Use of Single-Strand Conformation Polymorphism (SSCP) Analysis to Detect Mutations Leading to Clarithromycin-resistance in *Mycobacterium avium*." Graduate Research Development Project. March 1994 - December 1994.

Grant "Use of Random Amplified Polymorphic DNA (RAPD) Fingerprinting to Identify the Origin of Clarithromycin-resistant Isolates of *Mycobacterium avium*." Sigma-Xi Grant-in-Aid of Research. April 1993 - March 1994.

Invited Seminars

Graduate Teaching Assistant Workshop, Virginia Tech, Blacksburg, VA, August 19, 1996. *Title: Teaching a Life Sciences Laboratory*

E.O. Grundset Lecture Series, Southern College, Collegedale, TN, September 26, 1996. *Title: Clarithromycin-Resistance in Mycobacterium avium.*

Publications and Reports

Publications

Donna M. Jensen and Joseph O. Falkinham, III. **1996.** Drug Resistance Mechanisms in *Mycobacterium tuberculosis* and *Mycobacterium avium.* Recent Research Developments in Antimicrobial Agents and Chemotherapy **1**: 295-307.

Manuscripts Submitted

Donna M. Jensen-Cain, Laura E. Via, Constance A. Benson, Ann S. Vorys, and Joseph O. Falkinham, III. Origin of Macrolide-Resistant Isolates of *Mycobacterium avium* in AIDS Patients Undergoing Therapy. *Submitted to* : Antimicrob. Agents Chemother.

Donna M. Jensen-Cain and Joseph O. Falkinham, III. Identification of Clarithromycin-Resistant *Mycobacterium avium* Isolates Using the Polymerase Chain Reaction. *Submitted to* : J. Clinical Microbiol.

Published Abstracts

Donna M. Jensen, Laura E. Via, and Joseph O. Falkinham, III. **1994.** Random Amplified Polymorphic DNA (RAPD) Fingerprinting Identifies Spontaneous Clarithromycin-resistant Mutants in *Mycobacterium avium* - infected AIDS patients. **Plasmid.**

Donna M. Jensen and Joseph O. Falkinham, III. **1995.** Identification of Macrolide-Resistant *Mycobacterium avium* Strains Using the Polymerase Chain Reaction. **Plasmid.**

Abstracts Presented

Donna M. Jensen and Joseph O. Falkinham, III. Identification of Macrolide- and Azalide-Resistant *Mycobacterium avium* Isolates Using the Polymerase Chain Reaction. American Society for Microbiology General Meeting, New Orleans, LA, May 17-22, 1996.

Donna M. Jensen, Laura E. Via, and Joseph O. Falkinham, III. Spontaneous Macrolide-Resistant *Mycobacterium avium* Mutants Identified Using Random Amplified Polymorphic DNA (RAPD) Fingerprinting. Wind River Conference on Prokaryotic Biology in Estes Park, CO, June 4-8, 1994.

Donna M. Jensen and Joseph O. Falkinham, III. Isolation and Characterization of Clarithromycin-resistant Mutants of *Mycobacterium smegmatis.* Graduate Research Symposium on March 31, 1993, VPI & SU, Blacksburg, VA.

Professional Memberships

American Society for Microbiology (Member since 1993)
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Phi Kappa Phi -- elected member (Member since 1996)