A Single 16S Ribosomal RNA Substitution Is Responsible for Resistance to Amikacin and Other 2-Deoxystreptamine Aminoglycosides in *Mycobacterium abscessus* and *Mycobacterium chelonae*

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Twenty-six clinical isolates of *Mycobacterium abscessus* resistant to amikacin were identified. Most isolates were from patients with posttympanostomy tube placement otitis media or patients with cystic fibrosis who had received aminoglycoside therapy. Isolates were highly resistant (MICs >1024 μg/mL) to amikacin, kanamycin, gentamicin, tobramycin, and neomycin (all 2-deoxystreptamine aminoglycosides) but not to streptomycin. Sequencing of their 16S ribosomal (r) RNA revealed that 16 (94%) of 17 had an A→G mutation at position 1408. In vitro–selected amikacin-resistant mutants of *M. abscessus* and *Mycobacterium chelonae* had the same resistance phenotype, and 15 mutants all had the same A→G substitution at position 1408. Introducing an rRNA operon from *Mycobacterium smegmatis* with a mutated A→G at this position into a single functional allelic rRNA mutant of *M. smegmatis* produced the same aminoglycoside resistance phenotype. These studies demonstrate this 16S rRNA mutation is responsible for amikacin resistance in *M. abscessus*, which has only one copy of the rRNA operon.

Aminoglycosides have been a major component of therapy for mycobacterial diseases since the recognition of the remarkable activity of streptomycin against *Mycobacterium tuberculosis* in 1947 [1]. Currently, streptomycin is a second-line agent for the treatment of tuberculosis in developed countries and is often a first line agent in underdeveloped countries. Streptomycin is also used for therapy of some slow-growing nontuberculous *Mycobacterium* species, including *M. kansasii* [2], *M. xenopi* [3], *M. avium* complex [4], *M. terrae* complex, and *M. simiae* [5]. Its resistance among isolates of *M. tuberculosis* is complex, with high-level resistance associated with point mutations involving the ribosomal protein S12 (*rpsL* gene) and the 16S ribosomal RNA (rRNA) gene *(rrs)* [6–11]. The 16S rRNA gene region that is mutated involves a pseudoknot structure that is believed to be stabilized by the presence of the S12 protein [9]. Low-level resistance to streptomycin in *M. tuberculosis* is yet to be explained on the basis of genetic alterations [12] but is believed to result from mutations involving cell wall transport [7].

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Kanamycin and the closely related amikacin are used for treatment of multidrug resistant tuberculosis [13]. Amikacin is used routinely for treatment of serious infections due to rapidly growing *Mycobacterium* species, including *M. fortuitum* and *M. abscessus* [14–15], while tobramycin is the most active aminoglycoside for *M. chelonae* isolates [16]. Amikacin has also been used infrequently for the treatment of slow-growing nontuberculous mycobacterial diseases, including those due to *M. avium* complex [17, 18], *M. kansasii* [2], and *Mycobacterium haemophilum* [19]. The genetic basis of mycobacterial resistance to amikacin, kanamycin, and tobramycin has, until recently [20, 21], been unknown.

Phenotypic studies have shown that spontaneous mutants selected in the laboratory for resistance to amikacin, kanamycin, and tobramycin (2-deoxystreptamine aminoglycosides) are not cross-resistant to streptomycin for either *M. tuberculosis* [22] or the rapidly growing mycobacteria [23]. In addition, recent studies with *Mycobacterium smegmatis* have suggested that the 16S rRNA position 1408 (*Escherichia coli* numbering system) may be important for resistance to amikacin, tobramycin, and gentamicin [20, 21]. In the present study, we investigate 16S rRNA sequences for mutations in the rapidly growing mycobacterial species *M. chelonae* and *M. abscessus.*

Methods

Organisms. Isolates of *M. chelonae* and *M. abscessus* that were submitted to the Mycobacteria/Nocardia Laboratory at the University of Texas Health Center at Tyler for susceptibility testing between 1982 and July 1997 were screened for isolates resistant to the 2-deoxystreptamine aminoglycosides as defined below. Isolates

were taken from stock cultures frozen at −70°C at the time of susceptibility testing.

In vitro, spontaneous, resistant mutants of clinical isolates of *M. chelonae* and *M. abscessus* were selected by plating heavy suspensions of organisms on 100 μg/mL amikacin, tobramycin, and gentamicin and on 1000 μg/mL streptomycin. Single-colony mutants were subcultured, tested for aminoglycoside resistance, and then stored at −70°C. Details of these studies, mutational frequencies, and aminoglycoside MICs were previously published in 1985 [23]. Additional isolates were selected at a later date, using Luria-Bertani (LB) medium containing 50 μg/mL amikacin.

Clinical isolates were identified by standard growth and biochemical features, including the use of citrate as a sole carbon source for separation of *M. chelonae* and *M. abscessus* [24]. Isolates were also identified by susceptibility patterns [16] that included resistance to polymyxin [25] and differences in susceptibility to cefoxitin and tobramycin that separate *M. abscessus* from *M. chelonae* [16].

Clinical histories were obtained for patients with resistant strains at the time of submission for susceptibility testing.

Susceptibility testing. Susceptibility to amikacin, gentamicin, kanamycin, tobramycin, neomycin, and streptomycin was determined by use of the broth microdilution method in cation-supplemented Mueller-Hinton broth as previously described [26, 27]. Break points for resistance were those for the National Committee for Clinical Laboratory Standards (NCCLS) for organisms that grow aerobically [28]: amikacin, >32 μg/mL; kanamycin, >32 μg/mL; gentamicin, >8 μg/mL; and tobramycin, >8 μg/mL. No break points are available for neomycin and streptomycin. The broth microdilution method uses a final bacteria concentration of 10^4–10^5 cfu/mL in each well and an incubation period of 3 days at 30°C in room air.

Isolates subsequently shown to have the 16S rRNA A→G mutation at position 1408 were also tested for resistance to paromomycin at 200 μg/mL, using agar plates.

Pulsed-field gel electrophoresis (PFGE) and random arbitrarily primed DNA–polymerase chain reaction (RAPD-PCR). In circumstances where pretreatment or early treatment amikacin-susceptible and relapse amikacin–resistant isolate was available, DNA restriction fragment length polymorphism analysis of the 2 isolates was done by use of PFGE [29, 30] and RAPD-PCR [31].

For PFGE, cultures were taken from frozen stocks and subcultured to agar. PFGE was done as previously described for *M. abscessus* [29, 30]. In brief, organisms were taken from broth culture and cast into low melting–point agarose plugs and then lysed with 1 mg/mL lysozyme, 1% SDS, and 1 mg/mL proteinase K. Genomic DNA in the agarose plugs was digested by use of the infrequent cutting restriction endonucleases *Dra*I and *Xba*I and then separated by PFGE, using the CHEF Mapper system (Bio-Rad, Richmond, CA) at 14°C for 20 h at 6 V/cm.

RAPD-PCR was done as previously described [31]. In brief, cell suspensions were treated with lysozyme, proteinase K, and SDS. The lysates were extracted by phenol and chloroform, and DNA was precipitated by isopropanol. Fifty microliters of the mixture was used for PCR, which contained 60 m*M* Tris–HCL (pH 9.0); 2.5 m*M* $MgCl_2$; 15 m*M* $(NH_4)_2SO_4$; 250 μ*M* (each) dATP, dCTP, dGTP, and dTTP; 24 pmol of primer; 1 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis); and 2.5 μL of purified DNA (100 ng). The following primers were used: random primers, OPA2 (TGCCGAGCTG) and OPA18 (AGGTGACCGT), which were described by Kauppinen et al. [32], and primers INS-2 (GCGTAGGCGTCGGTGACAAA) and IS986-FP (ACGCTCAACGCCAGAGACCA), which were described by Linton et al. [33]. The amplification included 40 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The PCR products were separated by electrophoresis in a 2% agarose gel and detected after ethidium bromide staining.

16S rRNA DNA sequencing. For nucleic acid extraction, a small loopful of bacteria was dispersed in 100 μL H_2O, heated for 10 min at 80°C to inactivate the mycobacteria, and then transferred to a 1.5-mL screw-top plastic microfuge tube containing glass beads (100-μm diameter; Sigma, St. Louis). A tissue disintegrator (H. Mickle) was used to disrupt the cells. A 5-μL aliquot of the supernatant was used in PCR. Primers 283 (5′GAGTTTGATCCTGGCTCAGGA-3′, corresponding to 16S rRNA *E. coli* position 11–31) and 261 (5′AAGGAGGTGATCCAGCCGCA-3′, corresponding to 16S RNA *E. coli* positions 1539–1520) were used to amplify the 16S rRNA gene. Sequencing of 16S ribosomal DNA (rDNA) position 1408 was done with primer 289 (5′AAGTCGGGAGTCGCTAGTAAT-3′, corresponding to 16S rRNA *E. coli* position 1340–1349).

The nucleotide sequence of the amplified gene fragment was determined by direct sequencing. One of the primers (261) that was used in the amplification reaction was biotinylated to allow DNA sequencing using streptavidin-coated paramagnetic beads (Dynabeads; Dynal, Oslo).

Cloning of the rrnB operon of M. smegmatis. Standard methods were used for restriction endonuclease digestion of DNA and other manipulations [34]. Plasmid DNA isolation was either done by the alkaline lysis method or by use of the Qiagen preparation kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. All initial cloning procedures were performed with *E. coli* XL 1Blue MRF: △ *(mcrA)* 183, *(mcrCB-hsdSMR-mrr)* 173, *endA1, supE44, thi-1, recA1, gyrA96, relA1, lac (F′proAB, lac*^{q} Z△15), Tn*10(tet*^{r}) (Stratagene, La Jolla, CA).

The *rrnB* operon of *M. smegmatis* was isolated from a genomic library in λ ZapII (Stratagene) and identified by nucleic acid sequencing. After the phagemid was isolated, the *rrnB* operon (~6 kbp) was excised with *Sal*I and *Xba*I and cloned into the mycobacterial shuttle vector pMV261 [35], resulting in pMV261 rRNA.

PCR mutagenesis of 16S rRNA position 1408. To demonstrate that the A→G transition at position 1408 is responsible for the aminoglycoside-resistant phenotype in *M. chelonae* and *M. abscessus*, PCR techniques were used to mutate 16S rRNA position 1408 (A→G) in the cloned *rrnB* operon as described previously [21]. In addition, the 23S rRNA position 2058 was mutated (A→G) to allow for selection using clarithromycin. Sequence analysis confirmed that the respective mutations were introduced into the wild-type rRNA operon by this procedure.

Transformation of the PCR mutant plasmid construct of rrnB into a single allelic rRNA mutant of M. smegmatis. The cloned *rrnB* operon plasmid construct containing the mutated 16S rRNA 1408 A→G and 23S rRNA 2058 A→G was transformed into an *rrnB* mutant, a genetically engineered *M. smegmatis* rRNA knockout strain with a single functional rRNA operon [21] derived from strain *M. smegmatis* mc^2 155 [36]. The resultant *M. smegmatis* transformant would be heterodiploid with one chromosomal wild type 16S rRNA gene and a multicopy mutant 16S rRNA on the

pMV261 plasmid. Apparently the mutant 16S rRNA allele would be dominant under such circumstances.

Mycobacterial strains were made electrocompetent by use of the method described by Jacobs et al. [37], with the modification that bacteria were grown in brain-heart infusion medium (Oxoid, London) with 0.05% Tween 80. When an OD of 0.4 at A_{600} was achieved, cells were incubated on ice for 1.5 h. The cells then were collected by centrifugation (10 min, 5000 *g*, 4°C), washed with an equal volume of ice-cold glycerol (10%), and centrifuged.

Cells were subsequently resuspended in a one-tenth volume of the medium and again centrifuged at 5000 *g* for 10 min at 4°C. The pellet was resuspended in the same volume, and the cells were again centrifuged under the same conditions for 15 min. Finally, cells from 1 L of culture medium were resuspended in 2 mL of glycerol (10%). For electroporation, 100 μL of freshly prepared cells was mixed with 1 μg of DNA and put on ice for 5 min. Electroporation was performed in 0.2-cm cuvettes with a single pulse (2.5 kV, 25 μF, 1000 Ω; Bio-Rad). Cells were immediately resuspended in 1 mL of brain-heart infusion medium and incubated for 2 h with vigorous shaking at 37°C. Thereafter, primary selection was done on LB plates containing clarithromycin (50 μg/mL).

Results

Organisms. Twenty-six clinical isolates of *M. abscessus* resistant to amikacin (1/patient) were identified. There was no known relationship between any of the patients, and all were from different hospitals. The isolates fell into 3 disease groups. The first group comprised 14 ear isolates (54% of the strains) from patients with chronic otitis media following tympanostomy tube placement. One of these isolates was from a large outbreak [38]. The second group comprised 8 respiratory isolates (31%) from patients with chronic lung disease. The remaining 4 isolates (15%) were from patients with a variety of diseases, including endocarditis, catheter sepsis, and chronic wound infection. The majority of patients with pulmonary or miscellaneous infections (9/12 or 75%) were suspected or known to have underlying cystic fibrosis.

Almost all patients with available histories were known to have received prior aminoglycoside therapy. The patients with posttympanostomy otitis media had topical drops that usually contained neomycin, while most pulmonary patients had received either amikacin or tobramycin. The clinical summary of these 26 cases is shown in table 1. Clinical details for 9 patients with posttympanostomy tube placement otitis media (cases 1–5 and 8–11, table 1) [38, 39], 1 pulmonary case (case 15, table 1) [23], and 1 case of endocarditis (case 23, table 1) [23] have been published previously. No clinical isolates of aminoglycoside-resistant *M. chelonae* were identified.

Susceptibility testing. Aminoglycoside susceptibility results for the 26 clinical isolates of *M. abscessus* with amikacin resistance are shown in table 2. Most isolates had MICs of >1024 μg/mL to amikacin, kanamycin, tobramycin, gentamicin, and neomycin. Four patients had pretreatment or early treatment susceptible isolates (case nos. 16, 18, 20, and 23 in table 2) available for comparison, and these had typical MICs for untreated strains [16]. Susceptibility testing was not undertaken nor available for the other 22 pretreatment or early treatment case pairs. MICs for streptomycin were generally in the 64–256 μg/mL range for the amikacin-resistant strains (18/21), values which are comparable to currently observed and previously published values for wild-type strains [23]. MICs have been reported previously for 9 ear isolates [35, 36], 1 respiratory isolate (MC194) [23], and 1 blood isolate (MC92) [23].

Susceptibility results for the in vitro mutants of *M. chelonae* and *M. abscessus* are shown in table 3. Their MICs were comparable to clinical aminoglycoside-resistant isolates. Susceptibilities to paromomycin (neomycin E) were also determined for 8 *M. abscessus* mutants. The parental strains E-123 and ATCC 19977 had low MICs (<50 μg/mL), while the aminoglycoside-resistant mutants had MICs of >200 μg/mL to paromomycin. MICs for some of these isolates were reported previously [23].

PFGE and RAPD-PCR. There were 4 *M. abscessus* pretreatment or early treatment isolates as well as relapse isolates available for genetic comparison (case nos. 16, 18, 20, and 23 in tables 1 and 2). None produced intact genomic DNA after processing for PFGE, a problem previously noted with 50% of *M. abscessus* clinical isolates [29]. By RAPD-PCR and the use of three primers, however, each pretreatment susceptible strain and subsequent resistant strain from the same patient gave identical patterns, supporting that resistance is related to a change in the original susceptible infecting strain. A RAPD-PCR comparison of these strains using primer IS986-FP is shown in figure 1.

16S rRNA sequencing. Seventeen clinical isolates resistant to the 2-deoxystreptamine aminoglycosides underwent rDNA sequencing, with all but 1 (94%) having a guanine-for-adenine substitution at the 16S rRNA position 1408 (table 2; figure 2). Of four pretreatment or early treatment isolates that were susceptible to amikacin and the other 2-deoxystreptamine aminoglycosides, 3 were sequenced: All had a wild-type position 1408 (table 2, case nos. 16, 20, and 23). No substitution at position 1408 other than A→G was encountered among the resistant strains.

Similar sequencing results were observed among the in vitro–selected aminoglycoside-resistant mutants. Of the 7 mutants of *M. chelonae* that were resistant to the 2-deoxystreptamine aminoglycosides, all had the 1408 substitution. The 3 susceptible parent strains had a wild-type position 1408, as did a mutant selected for streptomycin resistance. Among 8 2-deoxystreptamine aminoglycoside–resistant mutants of *M. abscessus*, all 8 also had the 1408 mutation. Thus, overall, 15 of 15 in vitro, amikacin-resistant mutants of *M. chelonae* or *M. abscessus* had the 1408 A→G mutation, while 5 parent strains had a wild-type sequence.

PCR mutagenesis of 16S rRNA position 1408 with transformation into a single allelic rRNA mutant of M. smegmatis. The previously described *rrnB* operon was cloned into the replicating multicopy mycobacterial shuttle vector pMV261

Table 1. Clinical information on aminoglycoside-resistant isolates of *M. abscessus*.

Case no.	Organism no.	Source	Disease	Aminoglycoside exposure	Underlying disease
Posttympanostomy chronic ear disease					
1	349*	Ear	Otitis media, mastoiditis	Neomycin	None
2	434†	Ear	Otitis media	Neomycin‡	None
3	445*	Ear	Otitis media, mastoiditis	Neomycin	None
4	485*	Ear	Otitis media	Neomycin‡	None
5	514*	Ear	Otitis media	Neomycin, gentamicin	None
6	546	Ear	Otitis media	Unknown	None
7	543	Ear	Otitis media	Neomycin‡	None
8	890*	Ear	Otitis media, mastoiditis	Unknown	None
9	955*	Ear	Otitis media, mastoiditis, brain abscess	Neomycin‡	None
10	1002*	Ear	Otitis media, mastoiditis	Neomycin‡	None
11	1083*	Ear	Otitis media	Unknown	Unknown
12	1314	Ear	Otitis media	Tobramycin, multiple others	Epidermolysis bullosa
13	1661	Ear	Otitis media	Unknown	None
14	1820	Ear	Otitis media, mastoiditis	Neomycin‡	None
Chronic lung disease					
15	194§	Respiratory	Lung disease	Tobramycin	Cystic fibrosis
16	425	Respiratory	Lung disease	Amikacin	Bronchiectasis
17	876	Respiratory	Lung disease	Multiple others	Cystic fibrosis
18	1081	Respiratory	Lung disease	Tobramycin	Cystic fibrosis
19	1307	Respiratory	Lung disease	Unknown	Cystic fibrosis
20	1350	Respiratory	Lung disease	Unknown	(?) Cystic fibrosis
21	1535	Respiratory	Lung disease	Unknown	(?) Cystic fibrosis
22	1930	Respiratory	Lung disease	Unknown	Cystic fibrosis
Miscellaneous					
23	92§	Blood	Endocarditis	Amikacin	Catheter sepsis
24	630	Respiratory, blood	Lung disease, catheter sepsis	Tobramycin	Cystic fibrosis
25	967	Face	Abscess, infected nasolacrimal tube	Multiple	Severe local trauma, multiple corrections
26	1716	Skin, blood	Disseminated	Unknown	Lung transplant, cystic fibrosis

* Case previously reported [39].
† Case previously reported [38].
‡ Neomycin exposure is presumed since these patients all had history of topical ear drops (with neomycin the usual aminoglycoside present in drops).
§ Case previously reported [23].

and mutagenized at the 16S rRNA position 1408 (A→G) and at 23S rRNA position 2058 (A→G). This construct was then electroporated into *M. smegmatis* mc^2 155 KO-low, which has a single functional rRNA operon (knockout strain). Twelve transformants were selected from the clarithromycin-containing selection media. These isolates were all resistant to amikacin, tobramycin, and gentamicin, with MICs of >200 μg/mL, and to clarithromycin. As a control, a construct that was PCR mutagenized only at 23S rRNA position 2058 (A→G) was selected on the clarithromycin-containing medium and then tested for aminoglycoside resistance. Of 12 transformants, all were clarithromycin resistant but had wild-type aminoglycoside MICs (<50 μg/mL) to amikacin, tobramycin, and gentamicin.

Discussion

An initial study of aminoglycoside resistance in rapidly growing mycobacteria was published in 1985 [23]. This study demonstrated that high-level resistant mutants to amikacin (MIC >1024 μg/mL) could be regularly obtained in *M. chelonae* and *M. abscessus* (identified at that time collectively as *M. chelonei*) at a frequency of 10^{-5}–10^{-7}. These mutants were cross-resistant to other 2-deoxystreptamine aminoglycosides (gentamicin, tobramycin, and kanamycin) but produced no change in the MICs for streptomycin. High-level resistant mutants could be selected on any of the other 2-deoxystreptamine aminoglycosides with a similar frequency and the same cross-resistance pattern, demonstrating that all of the drugs were selecting an identical phenotype.

High-level streptomycin resistance in *M. abscessus* (MIC >1024 μg/mL) resulted in no change in the MICs to the 2-deoxystreptamine aminoglycosides. An aminoglycoside acetylating enzyme similar in its profile to aminoglycoside acetyltransferase (3)-III or -IV was evident in almost all (28/29 or 97%) pre-treatment susceptible isolates [40], with no change in detectable aminoglycoside-modifying enzymes in in vitro mutants or resistant strains recovered from patients on therapy.

Table 2. Aminoglycoside susceptibilities and results of sequencing of the 16S ribosome among clinical isolates of *M. abscessus*.

		Aminoglycoside MICs (μg/mL)						Position 1408	
Case no.	Organism no.	Amikacin	Kanamycin	Tobramycin	Gentamicin	Neomycin	Streptomycin	A → G mutation	WT
Posttympanostomy chronic ear disease									
1	349*	>1024	>1024	>1024	>1024	>1024	32, 64	+	−
2	434†	>1024	>1024	>1024	>256	>1024	ND	ND	ND
3	445*	>1024	>1024	>1024	>256	>1024	64	+	−
4	485*	>1024	>1024	>1024	>256	>1024	64	ND	ND
5	514*	>1024	>1024	>1024	>256	>1024	64	+	−
6	546	>32	ND	>32	ND	ND	ND	+	−
7	543	>32	ND	>32	ND	ND	ND	−	+
8	890*	>1024	>1024	>1024	>1024	>1024	128	+	−
9	955*	>1024	>1024	>1024	>1024	>1024	64	+	−
10	1002*	>1024	>1024	1024	512	1024	128	ND	ND
11	1083*	>1024	>1024	>1024	>1024	>1024	256	+	−
12	1314	>1024	>1024	>1024	>1024	>1024	128	+	−
13	1661	>1024	>1024	>1024	>1024	>1024	256	+	−
14	1820	>128	ND	>16	ND	ND	ND	ND	ND
Chronic lung disease									
15	194‡	>1024	>1024	>1024	>1024	ND	64	+	−
16	425 no. 1	16	8	32	128	64	256	−	+
	425 no. 2	>1024	>1024	>1024	>256	512	128	ND	ND
17	876	>1024	64	64	128	128	64	ND	ND
18	1081 no. 1	16	8	16	16	64	256	ND	ND
	1081 no. 6	>1024	>1024	>1024	>1024	>1024	256	ND	ND
19	1307	>1024	>1024	>1024	>1024	>1024	256	+	−
20	1350 no. 1	8	8	8	16	32	256	−	+
	1350 no. 2	>1024	>1024	>1024	>1024	>1024	>1024	ND	ND
21	1535	>1024	>1024	>1024	>1024	>1024	256	+	−
22	1930	>1024	>1024	>1024	>1024	>1024	256	ND	ND
Miscellaneous									
23	92 no. 1‡	8	4	16	16	ND	64	−	+
	92 no. 2‡	>1024	>1024	>1024	>1024	ND	128	+	−
24	630	>1024	>1024	1024	>256	>1024	4	+	−
25	967	>1024	>1024	512	>1024	256	512	+	−
26	1716	>128	ND	>16	ND	ND	ND	+	−

NOTE. WT = wild type; ND = not done.
* MICs previously reported except for streptomycin [39].
† MICs previously reported except for streptomycin [38].
‡ MICs previously reported [23].

No change in plasmid profiles was evident among resistant isolates. These findings supported a mutational event to explain the resistance, but genetic techniques to determine this were not yet available.

Since that study [23] was published >10 years ago, a great deal more has been learned about mycobacterial genetics. *M. chelonae* and *M. abscessus* are known to contain a single copy of the rRNA operon [30], making phenotypic expression of single point mutations in the ribosome much more likely than in most bacterial species that contain multiple copies. Mutational resistance to the newer macrolides, clarithromycin and azithromycin, was shown to result from a single point mutation at A-2058 or A-2059 (*E. coli* numbering system) in the 23S rDNA of *M. chelonae* and *M. abscessus* [30], as well as in other mycobacterial species with single genomic copies of the rRNA operon, such as *Mycobacterium intracellulare* [41] and *M. avium* [42, 43].

The first piece of evidence of the importance of the 16S rRNA position 1408 (*E. coli* numbering system) in aminoglycoside susceptibility was based on a study with an aminoglycoside-resistant strain of *Streptomyces tenjimariensis* that produced the little-known aminoglycoside istamycin. Following cloning of the resistance factor in *Streptomyces lividans* and demonstration that it was a methylase, Beauclerk and Cundiffe [44] showed that it methylated the adenine in 16S rRNA position 1408, with resultant high-level resistance to kanamycin and apramycin.

A second and more convincing piece of evidence of the importance of the 16S position 1408 in 2-deoxystreptamine aminoglycoside resistance was recently shown with studies with *M. smegmatis.* Using recombination techniques that allow for ''knockout'' of one of the two functional copies of the rRNA *(rrn)* operon in *M. smegmatis*, it was possible to select

Table 3. Aminoglycoside MICs and 16S rRNA position 1408 sequencing in in vitro mutants of *M. chelonae* and *M. abscessus*.

Strain,* Case no.	Aminoglycoside MICs (μg/mL)						Position 1408	
	Amikacin	Kanamycin	Tobramycin	Gentamicin	Neomycin	Streptomycin	A → G mutation	WT
M. chelonae								
68	32	16	4	32	32	128	−	+
AM-1	>1024	1024	>1024	>256	>1024	256	+	−
AM-2	>1024	1024	>1024	>256	>1024	256	+	−
KM-1	>1024	>1024	>1024	>256	>1024	256	+	−
KM-2	>1024	>1024	>1024	>256	1024	256	+	−
113	32	16	4	32	16	64	−	+
KM-2	>1024	>1024	1024	>256	>1024	64	+	−
109	16, 32	16, 32	2, 16	128	64	64, 256	−	+
AM-1	>1024	>1024	>1024	>256	1024	128, 512	+	−
AM-2	>1024	>1024	>1024	>256	>1024	512	+	−
SM-1	16, 64	8, 32	2, 16	128	64	>1024	−	+
M. abscessus								
E-123	<50	<50	ND	<50	ND	ND	−	+
AM-1010	>200	>200	ND	>200	ND	ND	+	−
AM-1011	>200	>200	ND	>200	ND	ND	+	−
AM-1012	>200	>200	ND	>200	ND	ND	+	−
AM-1013	>200	>200	ND	>200	ND	ND	+	−
ATCC 19977	<50	<50	ND	<50	ND	ND	−	+
AM-1015	>200	>200	ND	>200	ND	ND	+	−
AM-1016	>200	>200	ND	>200	ND	ND	+	−
AM-1017	>200	>200	ND	>200	ND	ND	+	−
AM-1018	>200	>200	ND	>200	ND	ND	+	−

NOTE. WT = wild type; ND = not tested.
* Mutants selected on amikacin (AM), kanamycin (KM), and streptomycin (SM).

gentamicin- or amikacin-resistant mutants in the knockout single allelic *rrn* strain at a rate of 10^{-8}. These single allelic *rrn* mutants were cross-resistant to other 2-deoxystreptamine aminoglycosides, and 10 of 10 mutants had the 16S rRNA 1408 A→G mutation [21]. We utilized a cloned *rrnB* operon that had been mutagenized at both 16S rRNA position 1408 (A→G) and at the 23S rRNA position 2058 (A→G), the latter being known to confer clarithromycin resistance, and inserted it into the extrachromosomally replicating, multicopy vector pMV 261. Transformation of the single *rrn* allelic mutant of *M. smegmatis* with this construct with selection on clarithromycin produced resistant transformants (12/12) with high-level amikacin resistance.

The final available evidence involved studies of amikacin- and kanamycin-resistant isolates of *Mycobacterium bovis* and *M. tuberculosis.* Studies of *M. bovis* bacille Calmette-Guérin, which has a single ribosomal operon, showed that 10 of 10 in vitro–selected mutants had this mutation, as did 8 of 8 clinical

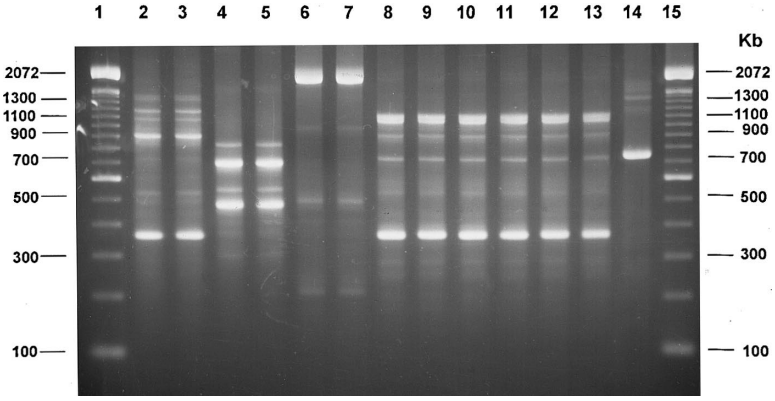

Figure 1. Arbitrarily primed polymerase chain reaction of isolates of *M. abscessus* from 4 patients before and after amikacin therapy using primer IS986-FP. Case 22 (table 1): lane 2, MC 92 no. 1 (pretreatment) and lane 3, MC 92 no. 2 (relapse); case 16: lane 4, MC 425 no. 1 (pretreatment) and lane 5, MC 425 no. 2 (relapse); case 20: lane 6, MC 1350 no. 1 (pretreatment) and lane 7, MC 1350 no. 2 (relapse); case 18: lane 8, MC 1081 no. 1 (pretreatment) and lanes 9–13, MC 1081 nos. 2–6 (during and after therapy). Lane 14, MC 1272 (control strain). Lanes 1 and 15, 100-bp DNA ladder.

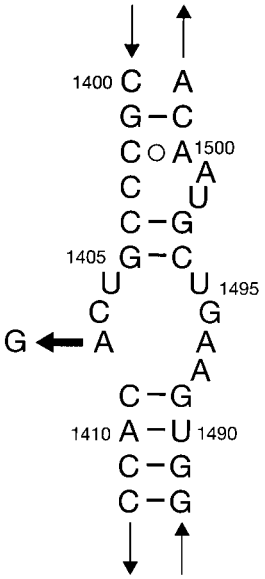

Figure 2. Secondary structure model of *Escherichia coli* 16S rRNA showing position 1408, which contains A→G mutation that confers resistance to amikacin and other 2-deoxystreptamine aminoglycosides.

isolates of amikacin- and kanamycin-resistant *M. tuberculosis* (which also has a single rRNA operon) [21]. These three pieces of evidence combined with the current study demonstrate that the substitution of guanine for adenine at position 1408 is the major mechanism of 2-deoxystreptamine aminoglycoside resistance in most mycobacterial species. Because of the small numbers of pretreatment (4) and resistant (17) clinical strains that were sequenced, we cannot exclude the possibility of genetic sites other than 16S rRNA position 1408 being involved in 2-deoxystreptamine aminoglycoside susceptibility. Additional studies may be required to help resolve this possibility.

The sequencing data in the current study and previous studies with other mycobacterial species [20] provided several surprises. The first was that a single base pair, position 1408, appeared to be responsible for most high-level resistance to amikacin (MIC >1024 μg/mL) and the other 2-deoxystreptamine aminoglycosides, including paromomycin. Presuming that the 2-deoxystreptamine aminoglycosides bind the 16S rRNA, this binding almost certainly involves multiple bases, but apparently mutation of only one is found in drug-resistant mutants.

Methylase genes conferring resistance at other sites on the 16S rRNA have been described (e.g., G-1405, which confers resistance to kanamycin plus gentamicin [44], has been identified from a strain of *Micromonospora purpurea* that produces gentamicin). At least four other sites (*E. coli* positions 1405, 1409, 1491, and 1495) within 16S rRNA have been identified at which mutations in other organisms cause resistance to aminoglycosides, such as paromomycin [44].

The second surprise was that substitution of only one of three potential nucleotides was observed (i.e., 1408 A→G) among clinical isolates and in vitro–selected *M. abscessus* mutants with a detectable mutation. With macrolide resistance, which involves A-2058 or A-2059 in the peptidyl transferase region of the 23S rRNA, base pair substitutions involving guanine, cytosine, and thymidine for adenine at both positions has been observed [41–43, 45–47]. Presumably the charge and structure of the 16S ribosome will only allow for a guanine substitution at this position.

More recently, the structure of the *E. coli* 16S rRNA complexed with the aminoglycoside antibiotic paromomycin was studied by nuclear magnetic resonance spectroscopy [48, 49]. Critical residues for paromomycin binding included the C1407-G1494 bp, A1408, A1493, and U1495 (*E. coli* numbering). An A1408–A1493 interaction was found essential for antibiotic binding as it led to the formation of a specific binding pocket. An interaction of equivalent geometry cannot be formed with a G1408–A1493 configuration, and paromomycin was found to bind only weakly to this mutant structure [49].

With in vitro–selected aminoglycoside-resistant mutants of *M. chelonae*, all had amikacin MICs of >1024 μg/mL when tested by broth microdilution MICs (7 mutants tested), all showed resistance to paromomycin (>200 μg/mL), and all had the position 1408 A→G mutation. In contrast, some in vitro–selected mutants of *M. abscessus* had amikacin MICs of 128–512 μg/mL, and position 1408 A→G mutations were often not detectable (data not shown). When heavy growth on all the 2-deoxystreptamine aminoglycosides in agar (200 μg/mL) was used as the basis for mutant selection, all of these isolates had the 1408 A→G mutation. This suggests that there is a group of *M. abscessus* isolates that have low-level 2-deoxystreptamine aminoglycoside resistance due to an alteration other than bp 1408. Of interest, all clinically resistant isolates of *M. abscessus* had high-level resistance, and all had the 1408 A→G mutation. A similar observation was recently made with *M. avium* strains resistant to clarithromycin [43] and with *M. tuberculosis* strains with low-level resistance to streptomycin [7].

A previous MIC study of in vitro 2-deoxystreptamine aminoglycoside–resistant mutants of *M. chelonae* and *M. abscessus* that included many of the isolates sequenced in the current study [23] demonstrated that these mutants showed little or no change in their MICs to streptomycin and that in vitro–selected, high-level streptomycin-resistant mutants showed no change in MICs for amikacin and the other aminoglycosides. The streptomycin MICs in the clinical amikacin-resistant isolates of *M. abscessus* in the current study were similar to those of untreated wild-type strains. The demonstration that high-level resistance to the 2 aminoglycoside groups is associated with different mutations (streptomycin with an *rpsL* or *rrs* mutation and the 2-deoxystreptamine aminoglycosides at position 1408 of the 16S rRNA) is consistent with the prior phenotypic observations.

The current study did not address aminoglycoside resistance in *M. fortuitum*, which is the other major pathogen among rapidly growing mycobacterial species. The previously published phenotype study [23] demonstrated that amikacin-resistant mutants could be selected in vitro on 10 μg/mL (frequency

of 10^{-5}–10^{-6}) but not on 100 μg/mL using parent strains with amikacin MICs of 1.0–2.0 μg/mL. These mutants had amikacin MICs of 8–32 μg/mL, with a comparable rise in MICs for the other 2-deoxystreptamine aminoglycosides. Utilizing these mutants, high-level resistant mutants to amikacin (MICs >1024 μg/mL) could then be achieved at a frequency of 10^{-6}. Amikacin-resistant isolates to *M. fortuitum* have been described [45, 46] but are relatively rare compared with those to *M. abscessus* (Wallace RJ, unpublished observations). *M. fortuitum* is known to contain two copies of the rRNA operon [47], unlike *M. chelonae* and *M. abscessus*, which only contain one [30]. How the previously observed phenotypic pattern of resistance relates to ribosomal mutations at position 1408 in this species has yet to be determined.

Because of the presence of a single copy of the ribosomal operon in the rapidly growing species *M. chelonae* and *M. abscessus*, single genetic events involving 16S rRNA position 1408 will result in high-level amikacin resistance. The mutational frequencies have been shown to be as high as 10^{-5} [23]. This suggests that aminoglycosides should not be used as monotherapy when large numbers of these organisms are present; this clinical approach was recommended before the genetic site of resistance was determined [4, 15]. Commonly used combination agents include clarithromycin and, with *M. abscessus*, cefoxitin.

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