ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1994, p. 1408–1412
0066-4804/94/$04.00+0

Vol. 38, No. 6

Tetracycline Resistance Determinants in *Mycobacterium* and *Streptomyces* Species

Y. PANG,[1] B. A. BROWN,[2] V. A. STEINGRUBE,[2] R. J. WALLACE JR.,[2] AND M. C. ROBERTS[1]*

Department of Pathobiology, University of Washington, Seattle, Washington 98195,[1] and Department of Microbiology, University of Texas Health Center, Tyler, Texas 75710[2]

Received 29 November 1993/Returned for modification 15 February 1994/Accepted 15 March 1994

Two of seven tetracycline-resistant (Tc^r) *Mycobacterium fortuitum* group isolates and six Tc^r clinical *Streptomyces* isolates carried gram-positive Tc^r determinants (Tet K and Tet L) and *Streptomyces* resistance determinants (Otr A, Otr B, and Otr C). This represents the first documentation of the aquisition by mycobacteria of determinants coding for antibiotic resistance and suggests the potential for the spread of antibiotic resistance determinants within mycobacterial species.

The nontuberculous mycobacteria and *Streptomyces* spp. are commonly found in soil. Many species in these groups, including *Mycobacterium marinum*, the pathogenic, rapidly growing mycobacteria (*Mycobacterium fortuitum* and *Mycobacterium chelonae*), and various *Streptomyces* spp., are capable of causing skin and skin-related infections following penetrating trauma (28). Prior to the 1970s, the potential of the tetracyclines (including doxycycline and minocycline) for use in the therapy of infections caused by these organisms was unknown. In 1981, Dalovisio et al. (5) reported successful treatment outcomes of skin and soft tissue infections caused by *M. fortuitum* in three patients treated with doxycycline alone and of an infection caused by *M. abscessus* (formerly *M. chelonae* subsp. *abscessus*) in one patient treated with doxycycline in combination with amikacin. Since then, there have been numerous reports of favorable treatment outcomes that were obtained when tetracyclines were used to treat soft tissue or bone infections caused by rapidly growing mycobacteria (21).

The in vitro tetracycline susceptibilities among the groups of pathogenic, rapidly growing mycobacteria are highly variable. Approximately 100% of the *Mycobacterium smegmatis* and 50% of the *M. fortuitum* (formerly *M. fortuitum* biovariant *fortuitum*) isolates are susceptible to doxycycline (MICs $\leq$4 μg/ml), whereas fewer than 25% of the *M. fortuitum* third biovariant complex, *M. abscessus* (formerly *M. chelonae* subsp. *abscessus*), and *M. chelonae* (formerly *M. chelonae* subsp. *chelonae*) are susceptible to doxycycline (24–27).

Recent increases in the number of cases of tuberculosis and diseases caused by the nontuberculous mycobacteria have been described (4, 8, 24, 25, 28). A limited number of active antimycobacterial agents are available, and little is known about the biochemical mechanisms and genetics of antimicrobial resistance in mycobacterial species, including resistance to tetracyclines.

Plasmids have been recognized in most species of nontuberculous mycobacteria, but they are not known to carry antibiotic resistance determinants (9, 11–13, 23). Antibiotic resistance toward isoniazid and rifampin in *Mycobacterium tuberculosis* has been linked to chromosomal mutations or deletions of existing genes (10, 22, 29), suggesting that changes in intrinsic genes are the primary (if not the only) basis of antibiotic resistance present in mycobacteria. In contrast, the most important type of resistance in other bacterial species has been the acquisition of new antibiotic resistance determinants, usually on mobile elements (15). A large number of tetracycline resistance (Tc^r) determinants (Tet determinants) are recognized in bacterial species, and most are associated with mobile plasmids or transposons (15, 20). Because of the importance of treating diseases caused by some nontuberculous mycobacteria with tetracycline and the potential importance of tetracyclines for treating diseases caused by actinomycetes (5, 21, 24, 26, 27), we evaluated seven Tc^r mycobacteria and six Tc^r *Streptomyces* spp. taken from among the clinical isolates submitted to the Texas Laboratory for susceptibility testing; we also evaluated a tetracycline-susceptible (Tc^s) reference strain (Table 1). Bacterial isolates with the following genes and plasmids were hybridized with the indicated tetracycline resistance gene probes: *tetK* (pAT102), 870-bp *Hin*cII probe; *tetL* (pVB11.15), 3.7-kb *Hin*cII probe; *tetM* (pJI3), 5-kb *Hin*cII probe; *tetO* (pUOA4), 1.8-kb *Hin*cII probe; *Bacteroides tetQ* (pNFD13-2), 0.9-kb *Eco*RI-*Eco*RV probe; and *tetB* (pRT11), 1.27-kb *Hin*cII probe (3, 15, 20). Oxytetracycline-producing *Streptomyces* spp. obtained from environmental sources have been shown to have oxytetracycline resistance determinants linked to their oxytetracycline biosynthetic pathways. The determinants included Otr A (pGLW7), a 2.5-kb *Bam*HI-*Sac*I probe; Otr B (pGLW10), a 3-kb *Pst*I-*Sac*I probe; and Otr C (pGLW52), a 6-kb *Kpn*I probe (2, 6, 7). Otr A and Otr B have the same mechanisms of resistance as the Tet determinants; Otr A codes for proteins which protect the ribosomes, and Otr B has an active efflux system (6, 7), while the mechanism for Otr C is unknown. Hybridization with radiolabelled DNA was done under stringent conditions of 42°C with 50% (vol/vol) formamide, 0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) albumin, 0.1% (wt/vol) Ficoll, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 0.05 M monobasic sodium phosphate (pH 7.4), 0.005 M EDTA, 0.76 M NaCl, and 200 μg of boiled calf thymus DNA per ml overnight (16, 17). The filters were then washed three times for 10 min each time at 52°C in 0.1% SDS–0.015 M NaCl–0.0015 M sodium citrate; this was followed by three 10-min washes at 52°C in 0.015 M NaCl–0.0015 M sodium citrate. These conditions required >80% sequence identity between the template and labelled DNA for a positive hybridization reaction (16). PCR assays were done for Otr A, Otr B, Tet K/L, and Tet M/O. The Tet K/L PCR assay used 2 U of *Taq* polymerase (Perkin-

* Corresponding author. Mailing address: Department of Pathobiology SC-38, University of Washington, Seattle, WA 98195. Phone: (206) 543-8001. Fax: (206) 543-3873. Electronic mail address: marilynr@u.washington.edu.

TABLE 1. Characteristics of the isolates examined in the present study

Isolate	Source	Year of isolation	Geographic location	Clinically significant	MIC (μg/ml)[a]			Tet determinants[b]	
					Tc	Doxy	Mino	Otr	Tet
Mycobacterium fortuitum									
ATCC 6841	Cold abscess	1936	Brazil	Yes	≤1	≤0.25	0.5	Neg	Neg
307	Sternal wound	1985	Texas	Yes	>16	4	1	Neg	Neg
414	Breast implant	1986	Florida	Yes	>16	16	4	Neg	Neg
Mycobacterium fortuitum third biovariant complex									
ATCC 49403	Facial abscess	1980	Texas	Yes	8	>32	>16	A, B	K
ATCC 49404	Infected scalp	1987	Louisiana	Yes	>16	>16	4	Neg	Neg
744	Stump incision	1990	Florida	Yes	>16	4	4	Neg	Neg
Mycobacterium peregrinum									
ATCC 14467	Bronchial aspirate	1962	Mexico	?[c]	>16	16	2	A, B	K, L
494	Foot	1987	Texas	Yes	>16	8	2	Neg	Neg
Streptomyces sp. strain									
5	Foot	1978	Texas	?	>16	4	2	A, B	K, L
32	Conjunctiva	1978	Texas	?	8	4	2	A, B, C	K
41	Peripheral blood	1989	Texas	?	16	8	2	A, B, C	K
42	Blood	1990	Texas	No	>16	16	8	A, B, C	K, L
43	Finger	1990	Texas	Yes	8	4	1	A, B	K, L
44	Lung wash (lung cancer)	1990	Texas	No	16	8	2	A, B, C	K

[a] Tc, tetracycline; Doxy, doxycycline; Mino, minocycline. Susceptible, ≤4 μg of tetracycline per ml; intermediate, 8 μg of tetracycline per ml; resistant, ≥16 μg of tetracycline per ml Tc (21, 24).
[b] Neg, negative for hybridizations with Tet B, Tet K, Tet L, Tet M, and Tet O or Otr A, Otr B, and Otr C.
[c] ?, inadequate history for evaluation.

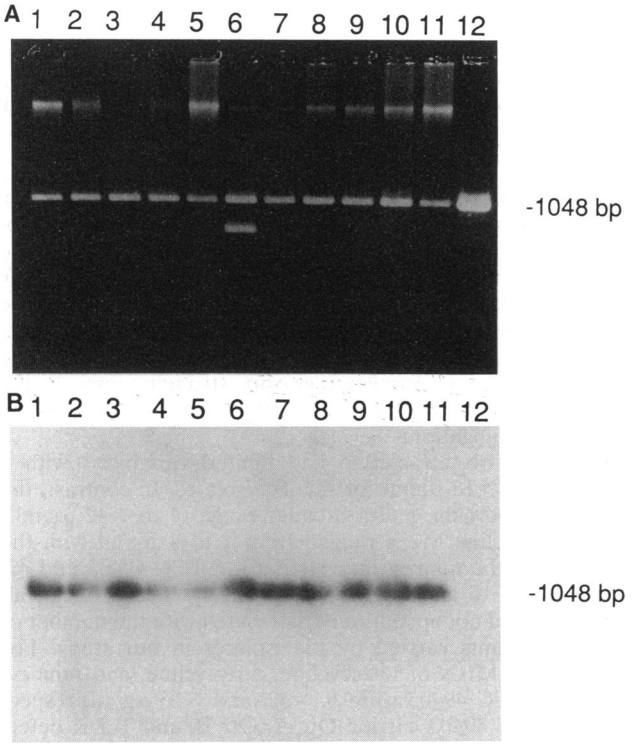

FIG. 1. (A) Gel electrophoresis of the 1,048-bp Tet K/L PCR products. The assay detected Tet K and/or Tet L determinants. However, the fragment produced was specific for Tet K or Tet L; thus, the Tet K fragment did not hybridize with the Tet L probe, and vice versa (14a). Lane 1, control carrying Tet K; lane 2, *M. peregrinum* ATCC 14467; lane 3, *M. fortuitum* third biovariant ATCC 49403; lane 4, control carrying Tet K; lanes 5 to 10, streptomyces 41, 43, 44, 32, 5, and 42, respectively; lane 11, plasmid pAT102 [cloned Tet K]; lane 12, plasmid pUB11.15 [cloned Tet L]. (B) Southern hybridization with the ^{32}P-labeled Tet K fragment. Lanes 1 to 10 were positive for Tet K. The Tet L PCR product in lane 12 did not hybridize with the labelled Tet K DNA under stringent conditions, although they shared 65 to 70% DNA homology (15), nor did the lower band in lane 6 hybridize with the labeled Tet K DNA.

Elmer Cetus, Norwalk, Conn.), 200 μM (each) deoxynucleoside triphosphates, 1× PCR buffer II (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.001% [wt/vol] gelatin), 2.5 mM $MgCl_2$, 50 ng of each oligonucleotide, and 20 ng of *Mycobacterium* or *Streptomyces* DNA as template in a total reaction volume of 30 μl. Reactions were conducted by using a Perkin-Elmer Cetus DNA thermal cycler for 35 cycles, each cycle of which consisted of 96°C for 15 s, 45°C for 30 s, and 72°C for 2 min (14a). Similar conditions were used for the Otr A and Otr B PCR assays, while the Tet M/O PCR assay was done as described previously (18). The PCR assays were used to verify the hybridization results obtained with the labelled tetracycline and oxytetracycline resistance gene probes. The primers from the four PCR assays were labelled with the Genius system (Boehringer-Mannheim, Indianapolis, Ind.) and were hybridized by the manufacturer's protocol to provide a third method of verifying the presence of the Tet K, Tet L, Otr A, and Otr B determinants.

Consistent with its phenotype, the Tc^s reference mycobacterium did not hybridize with any of the DNA or oligonucleotide probes, nor did it give PCR products (16, 18). All six of the Tc^r *Streptomyces* spp. hybridized with two or three of the Otr determinants tested (Table 1); this represents the first description of the presence of Otr determinants in clinical

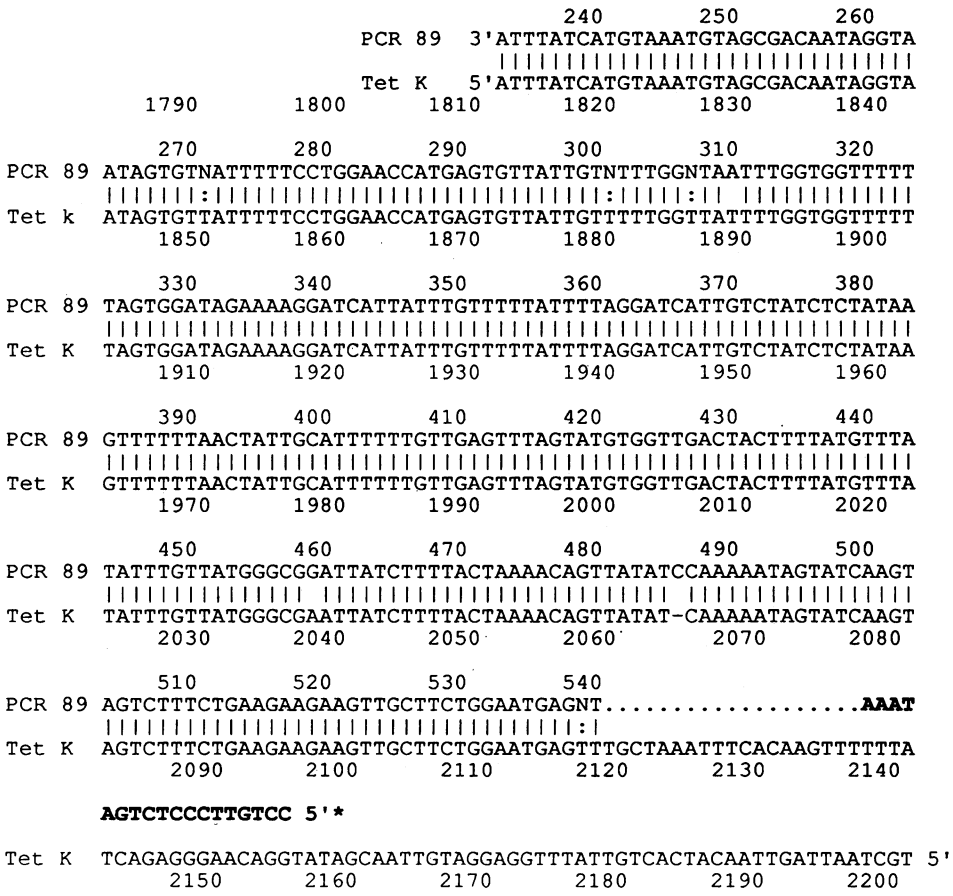

FIG. 2. Nucleotide sequence comparison of the *tetK* gene from pT181 [labelled Tet K] from *Staphylococcus aureus* and the PCR sequence from *M. fortuitum* third biovariant ATCC 49403 (labeled PCR 89). *, the primer sequence (in boldface type); N, the base pair could not be determined.

Streptomyces isolates. Unexpected was the finding of Otr A and Otr B in two (29%) of the seven Tc^r mycobacteria (Table 1). Tet K and Tet L were detected in the same two mycobacteria and all six *Streptomyces* spp. (Table 1; Fig. 1). The PCR product from the *M. fortuitum* third biovariant complex ATCC 49403 was extracted by using an extraction kit (Qiagen, Chatsworth, Calif.) and was sequenced with a sequencing kit (Applied Biosystems, Foster City, Calif.) on model 373A sequencer (Applied Biosystems). The Tet K primers amplified a sequence spanning nucleotides 1110 to 2140 of *tetK* from pT181 (GenBank accession number J01764). The first 300 bp of the PCR-generated sequence was matched between nucleotides 1820 and 2120 of the Tet K determinant from pT181 and had 98% identity (Fig. 2). A similar degree of identity was seen with the Tet K determinant from plasmid pNS1 (GenBank accession number M16217) (data not shown). As with all other *tetK* and *tetL* sequences, the sequence from *M. fortuitum* had a G+C content of approximately 35%, which is significantly different from the approximate 70% G+C content found in streptomyces and mycobacteria (14, 15, 19). Previously, we have found the Tet L determinant in members of the genera *Actinomyces*, *Clostridium*, *Enterococcus*, *Listeria*, *Peptostreptococcus*, and *Streptococcus* (15, 16), while other investigators have found Tet L in members of the genera *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Streptococcus* (20). Similarly, the Tet K determinant is associated with members of the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Listeria*, *Peptostreptococcus*, *Staphylococcus*, and *Streptococcus* (15, 16, 20). However, this report represents the first time that either of these determinants has been described in members of the genera *Mycobacterium* and *Streptomyces*. Because the Tet K and Tet L determinants have a low G+C content, can be transferred by conjugation to other species, and are found in a number of gram-positive species (15, 16, 20), their presence in *Mycobacterium* and *Streptomyces* spp. strongly supports the hypothesis that they were acquired from other gram-positive species by conjugation.

The MICs of tetracycline had limited variability, with a range of 8 to >16 μg/ml for the Tc^r isolates. In contrast, the MICs of doxycycline had a broader range (4 to >32 μg/ml), while minocycline MICs ranged from 1 to 4 μg/ml with the exception of the minocycline MICs for ATCC 49403 and 42, which were >16 and 8 μg/ml, respectively. The MICs of all three drugs did not appear to be associated with the number of Tc^r determinants carried by the isolates in our study. For example, the MICs of tetracycline, doxycycline, and minocycline for ATCC 49403 were 8, >32, and >16 μg/ml, respectively; [ATCC 49403 carried Otr A, Otr B, and Tet K determinants], while the MICs of tetracycline, doxycycline, and minocycline for ATCC 14467 were >16, 16, and 2 μg/ml, respectively [ATCC 14467 carried four determinants: Otr A, Otr B, Tet K and Tet L].

Current data suggest that antibiotic-producing *Streptomyces* spp. may be the ancestral source for many of the antibiotic

resistance determinants found in other genera (1). It is an interesting twist that these bacteria are now the likely source of the Tet K and Tet L determinants found in Tc^r *Streptomyces* spp. and some of the Tc^r mycobacteria described in this report. The *Streptomyces* spp. and mycobacteria have followed the trend of other bacterial species in carrying more than one *tet* gene (16).

Most bacteria become clinically resistant to antibiotics by acquiring new determinants which are then capable of moving to other isolates and species (15). The transfer frequencies of these determinants are usually greater when both the donor and the recipient are of the same or related species (15). *Streptomyces* spp. are gram-positive bacteria with traits related to those found in the genus *Mycobacterium*, and members of the two genera share the same habitat (14, 19). This may explain why the two mycobacteria acquired the Otr determinants. However, six of the Tc^r mycobacteria did not hybridize with any of the Tet or Otr determinants tested. We have seen this with other Tc^r bacteria (16) and hypothesize that different or novel Tet determinants are present in the isolates which did not hybridize with any of the determinants tested.

The data presented here suggest the potential for the spread of an antibiotic resistance gene into all environmental (nontuberculous) mycobacteria, including *Mycobacterium leprae*. Therefore, monitoring of mycobacteria for the development of Tc^r should be considered. In addition, rRNA methylase determinants, which confer resistance to lincosamides and the new macrolides, are often associated with the Tet determinants (3, 15). Use of the new macrolides in the treatment and, possibly, the prophylaxis of disseminated *Mycobacterium avium* complex infections in patients with AIDS and patients with disease caused by rapidly growing mycobacterial (24, 27) could provide the pressure to introduce one of these mobile methylase determinants as well as enhance the spread of the Tet determinants into mycobacterial species.

M.C.R. was supported by Public Health Service grant AI24136 from the National Institutes of Health.

We thank I. S. Hunter for the Otr probes and H. Wang for help with the sequencing.

REFERENCES

1. **Benveniste, R., and J. Davies.** 1973. Aminoglycoside antibiotic-inactivation enzymes in actinomycetes similar to those present in clinical isolates of antibiotic resistant bacteria. Proc. Natl. Acad. Sci. USA **172**:3628–3632.
2. **Butler, M. J., E. J. Friend, I. S. Hunter, F. S. Kaczmarek, D. A. Sugden, and M. Warren.** 1989. Molecular cloning of resistance genes and architecture of a linked gene cluster involved in biosynthesis of oxytetracycline by *Streptomyces rimosus*. Mol. Gen. Genet. **215**:231–238.
3. **Clewell, D. B., and C. Gawron-Burke.** 1986. Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Annu. Rev. Microbiol. **40**:635–659.
4. **Collins, F. M.** 1993. Tuberculosis: the return of an old enemy. Crit. Rev. Microbiol. **19**:1–16.
5. **Dalovisio, J. R., G. A. Pankey, R. J. Wallace, and D. B. Jones.** 1981. Clinical usefulness of amikacin and doxycycline in the treatment of infection due to *Mycobacterium fortuitum* and *Mycobacterium chelonei*. Rev. Infect. Dis. **3**:1068–1074.
6. **Dittrich, W., and H. Schrempf.** 1992. The unstable tetracycline resistance gene of *Streptomyces lividans* 1326 encodes a putative protein with similarities to translational elongation factors and Tet(M) and Tet(O) proteins. Antimicrob. Agents Chemother. **36**:1119–1124.
7. **Doyle, D., K. J. McDowall, M. J. Butler, and I. S. Hunter.** 1991. Characterization of an oxytetracycline-resistance gene, *otrA*, of *Streptomyces rimosus*. Mol. Microbiol. **5**:2923–2933.
8. **Edlin, B. R., J. I. Tokars, M. H. Grieco, J. T. Crawford, J. Williams, E. M. Sordillo, K. R. Ong, J. O. Kilburn, S. W. Dooley, K. G. Castro, W. R. Jarvis, and S. D. Holmberg.** 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. **326**:1514–1521.
9. **Hermans, J., C. Marin, G. N. M. Huijberts, T. Goosen, and J. A. M. de Bont.** 1991. Transformation of *Mycobacterium aurum* and *Mycobacterium smegmatis* with the broad host-range gram-negative cosmid vector pJRD215. Mol. Microbiol. **5**:1561–1566.
10. **Heym, B., and S. T. Cole.** 1992. Isolation and characterization of isoniazid-resistant mutants of *Mycobacterium smegmatis* and *M. avium*. Res. Microbiol. **143**:721–730.
11. **Hillyer, T. J., I. N. Brown, J. W. Dale, and C. S. F. Easmon.** 1991. Plasmid analysis of *Mycobacterium avium-intracellulare* (MAI) isolated in the United Kingdom from patients with and without AIDS. J. Med. Microbiol. **34**:225–231.
12. **Hull, S. I., R. J. Wallace, Jr., D. G. Bobey, K. E. Price, R. A. Goodhines, J. M. Swenson, and V. A. Silcox.** 1984. Presence of aminoglycoside acetyltransferase and plasmids in *Mycobacterium fortuitum*: lack of correlation with intrinsic aminoglycoside resistance. Am. Rev. Respir. Dis. **129**:614–618.
13. **Lazraq, R. S., S. Clavel-Seres, H. L. David, and D. Roulland-Dussoix.** 1990. Conjugative transfer of a shuttle plasmid from *Escherichia coli* to *Mycobacterium smegmatis*. FEMS Microbiol. Lett. **69**:135–138.
14. **Locci, R.** 1989. Streptomycetes and related genera, p. 2451–2491. *In* S. T. Williams, M. S. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 4. The Williams & Wilkins Co., Baltimore.

14a. **Pang, Y., et al.** Unpublished data.

15. **Roberts, M. C.** 1989. Gene transfer in the urogenital and respiratory tract, p. 347–375. *In* S. Levy and R. V. Miller (ed.), Gene transfer in the environment. McGraw-Hill Book Co., New York.
16. **Roberts, M. C., and S. L. Hillier.** 1990. Genetic basis of tetracycline resistance in urogenital bacteria. Antimicrob. Agents Chemother. **34**:261–264.
17. **Roberts, M. C., R. B. Leonard, A. M. Briselden, F. D. Schoenknecht, and M. B. Coyle.** 1992. Characterization of antibiotic resistant *Corynebacterium striatum* strains. J. Antimicrob. Chemother. **30**:463–474.
18. **Roberts, M. C., Y. Pang, D. E. Riley, S. L. Hillier, R. C. Berger, and J. N. Krieger.** 1993. Detection of Tet M and Tet O tetracycline resistance genes by polymerase chain reaction. Mol. Cell. Probes **7**:387–393.
19. **Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.).** 1986. *In* Bergey's manual of systematic bacteriology, vol. 2, p. 1435–1459. The Williams & Wilkins Co., Baltimore.
20. **Speer, B. S., N. B. Shoemaker, and A. A. Salyers.** 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Rev. Microbiol. **5**:387–399.
21. **Swenson, J. M., C. Thornsberry, and V. A. Silcox.** 1982. Rapidly growing mycobacteria: testing of susceptibility to 34 antimicrobial agents by broth microdilution. Antimicrob. Agents Chemother. **22**:186–192.
22. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer.** 1993. Detection of rifampicin-resistant mutations in *Mycobacterium tuberculosis*. Lancet **i**:647–650.
23. **Udou, T., Y. Mizuguchi, and R. J. Wallace, Jr.** 1987. Patterns and distribution of aminoglycoside-acetylating enzymes in rapidly growing mycobacteria. Am. Rev. Respir. Dis. **136**:338–343.
24. **Wallace, R. J., Jr., B. A. Brown, and G. O. Onyi.** 1992. Skin, soft tissue, and bone infections due to *Mycobacterium chelonae chelonae*: importance of prior corticosteroid therapy, frequency of disseminated infections, and resistance to oral antimicrobials other than clarithromycin. J. Infect. Dis. **166**:405–412.
25. **Wallace, R. J., Jr., B. A. Brown, and V. A. Silcox.** 1991. Clinical disease, drug susceptibility and biochemical patterns of the unnamed third biovariant complex of *Mycobacterium fortuitum*. Infect. Dis. **163**:598–603.
26. **Wallace, R. J., Jr., D. R. Nash, M. Tsukamura, Z. M. Blacklock,**

and V. A. Silcox. 1988. Human disease due to *Mycobacterium smegmatis*. J. Infect. Dis. **158:**52–59.

27. **Wallace, R. J., Jr., J. M. Swenson, V. A. Silcox, and M. G. Bullen.** 1985. Treatment of nonpulmonary infection due to *Mycobacterium fortuitum* and *Mycobacterium chelonae* on the basis of in vitro susceptibility. J. Infect. Dis. **152:**500–514.
28. **Wallace, R. J., Jr., J. M. Swenson, V. A. Silcox, R. C. Tschen, and M. S. Stone.** 1983. Spectrum of disease due to rapidly growing mycobacteria. Rev. Infect. Dis. **5:**657–679.
29. **Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole.** 1992. The catalase-peroxidase gene and isoniazid resistance of *M. tuberculosis*. Nature (London) **358:**591–593.