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Distribution of Environmental Mycobacteria in Karonga District, Northern Malawi

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The genus *Mycobacterium* includes many species that are commonly found in the environment (in soil and water or associated with plants and animals), as well as species that are responsible for two major human diseases, tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*). The distribution of environmental mycobacteria was investigated in the context of a long-term study of leprosy, tuberculosis, *Mycobacterium bovis* BCG vaccination, and the responses of individuals to various mycobacterial antigens in Karonga District, northern Malawi, where epidemiological studies had indicated previously that people may be exposed to different mycobacterial species in the northern and southern parts of the district. A total of 148 soil samples and 24 water samples were collected from various locations and examined to determine the presence of mycobacteria. The detection method involved semiselective culturing and acid-fast staining, following decontamination of samples to enrich mycobacteria and reduce the numbers of other microorganisms, or PCR with primers specific for the mycobacterial 16S rRNA gene, using DNA extracted directly from soil and water samples. Mycobacteria were detected in the majority of the samples, and subsequent sequence analysis of PCR products amplified directly from soil DNA indicated that most of the products were related to known environmental mycobacteria. For both methods the rates of recovery were consistently higher for dry season samples than for wet season samples. All isolates cultured from soil appeared to be strains of *Mycobacterium fortuitum*. This study revealed a complex pattern for the environmental mycobacterial flora but identified no clear differences between the northern and southern parts of Karonga District.

Soil bacterial populations are large and diverse and are influenced by abiotic factors, such as climate and soil type, as well as by local vegetation and other biotic inputs. Like many other groups of bacteria, some *Mycobacterium* species are common soil and water inhabitants, and these organisms are often referred to as "environmental mycobacteria." In contrast, the causative agents of tuberculosis (TB) and leprosy (*Mycobacterium tuberculosis* and *M. leprae*, respectively) and *M. bovis*, the origin of the attenuated BCG vaccine used against TB, are obligate parasites, as well as human and animal pathogens. Some environmental mycobacteria (EM) have been implicated in human infections, particularly in immunocompromised patients, who may be exposed through inhalation, ingestion, or broken skin (23). Understanding the sources, frequency, and consequences of human exposure to these microorganisms is also important because immune responses following exposure to EM may influence susceptibility to tuberculosis and leprosy and may even block the proliferation and protective effect of BCG vaccination against tuberculosis (3). Environmental mycobacterial exposure is thus considered the main reason for the great variation in protection against TB (from 0 to 80%) provided by BCG vaccination in different regions of the world (8, 15, 21).

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Investigation of EM in soil is limited by the lack of appropriate methods. Culturing can be used to screen samples from raw and treated water systems, which may harbor opportunistic pathogen species, such as *M. avium* and *M. intracellulare* (7). However, laboratory protocols that are commonly used to investigate the presence of mycobacteria in clinical specimens, including methods for decontamination of samples by treatment with biocides (to inhibit or eliminate the majority of microorganisms under conditions in which most mycobacteria survive), selective culturing, DNA extraction, and PCR, are inefficient for soil and natural water samples. Most of the mycobacteria isolated from soil are "fast-growing" species, although some slow growers, including *M. avium*, have been reported (13, 14). Compared to clinical specimens, soil has a wider and less predictable pH range and contains variable quantities of organic and inorganic materials, including cell-free enzymes, heavy metals, and trace elements, as well as many nonmycobacterial microorganisms that can withstand the selective decontamination and culture procedures and thereby degrade the culture medium and inhibit PCR detection. Mycobacteria have waxy, lipid-rich cell walls that are relatively resistant to biocides used in decontamination procedures and also provide the characteristic "acid-fast" property when they are treated with Ziehl-Neelsen (ZN) stain. The staining intensity varies according to the growth stage and species, and some nonmycobacteria that occur in soil, including some *Nocardia* spp., are also weakly acid fast. Many of the primers designed for mycobacterial detection with PCR are aimed at pathogenic species in clinical specimens. A comparison of 16S rRNA gene sequences enabled

workers to design genus-specific primers that amplify a 473-bp section that varies in many EM, although not in pathogens related to *M. tuberculosis* (20).

The Karonga Prevention Study (KPS) is a large research program based in Karonga District, a rural area in northern Malawi, which has been devoted primarily to studies of the natural history and control of tuberculosis and leprosy. Several observations have indicated that EM have important effects in these diseases. Routine culture of sputum specimens from suspected tuberculosis patients has revealed that the sputum commonly contains EM (10). Skin test and gamma interferon response surveys have provided evidence that there is widespread but differential exposure to and sensitization by a variety of EM antigens (2, 10) and have suggested that exposure to certain antigens of fast-growing EM is associated with a reduced risk of leprosy and tuberculosis (10). The incidence of leprosy has been threefold higher in the northern part of the district, which is hotter and wetter, than in the southern part of the district, whereas skin test data have suggested that there is twofold-greater exposure to cross-reacting EM in the southern part of the district (9). Finally, BCG vaccination appears to provide little protection against tuberculosis in Karonga District, which may reflect masking of protection through exposure to an EM (8, 15).

The purpose of the study reported here was to survey EM in soil and water in Karonga District in order to determine if there were any obvious differences in the prevalence of the dominant EM which might account for differences in human exposure in the northern and southern parts of the district. This was done by comparing EM in samples obtained from similar locations in the northern and southern parts of the district and in the wet and dry seasons. The utility of decontamination-culture methods and the utility of PCR-based methods were compared, and the genetic diversity of isolates and PCR-amplified fragments was examined.

MATERIALS AND METHODS

Characteristics of the study site. Karonga District is located in the northern part of Malawi in southern Africa, at 9° to 10°S latitude and 33° to 34°E longitude, between the high ground of the Nyika Plateau (elevation, approximately 2,100 m) to the west and Lake Malawi to the east. The district typically experiences a warm and wet season from December to April (average temperature, 24°C), a cool and dry season from May to August (21°C), and a hot and dry season from September to November (25°C). The mean annual rainfall is >1,600 mm in the north and 800 to 1,200 mm in the south. The soil has been reported to be generally loamy and acidic to neutral in the north and more sandy and acidic to alkaline farther south; for samples collected for this project the mean pH was 5.99 (standard error, 0.13) in the north and 6.7 (standard error, 0.26) in the south. Similar sites in villages and households in the northern and southern parts of the district were selected for sampling. The households included households in which individuals who had EM isolated from sputum samples had resided; in the majority of cases, the isolates had been identified as *M. avium.*

Collection of soil and water samples. Soil samples were collected from bathing enclosures, kitchens, bare ground, and animal enclosures belonging to 19 households in 11 villages with a history of multiple cases of leprosy or TB, as well as from river banks and the immediate surroundings of three health centers. At each sampling position, a grid that was 10 m by 10 m (or 6 m by 6 m where space was limited) was drawn. Spots were marked 1.5 m apart along crossing lines drawn from corner to corner inside each grid. A soil corer was used to collect approximately 2 g of soil from 5 to 25 cm below the surface at each marked spot. The surface layer was not collected because this layer is subject to rapid environmental change, including sterilization by solar radiation, in contrast to the subsurface soil; the aim was to collect soil microbial populations representative of the site. All subsamples from the same grid were placed into the same sterile plastic bag to make one composite sample. The corer was cleaned with a swab saturated with 95% ethanol when it was moved from one sampling grid to another. A total of 113 composite soil samples were collected at the end of the dry season in 1998. A total of 35 composite soil samples were collected at the end of the wet season in 1999 from 10 households; 32 of these samples were from sampling positions where soil had been collected in the dry season.

Water samples were collected from drinking water taps, boreholes, and ground wells associated with the households. Twenty samples were collected at the end of the dry season in 1998, and 10 repeat samples were collected from the same sources at the end of the rainy season in 1999. Samples from communal taps and boreholes were collected using sterile 250-ml or 500-ml plastic bottles (Bibby Sterilin). Samples from shallow wells and rivers were collected directly in bottles with the open mouth facing forward; with one forward hand movement, each bottle was filled to the 500-ml mark. Immediately after collection, all samples were placed in a cool box containing ice packs (4 to 10°C) for transport to the laboratory, where they were stored at 4°C.

Direct detection of mycobacteria in soil and water samples using PCR. Microbial DNA was extracted directly from 250-mg portions of the composite soil samples using a MoBio Laboratories Ultraclean soil DNA kit according to the manufacturer's protocol, which resulted in 50-μl soil DNA solutions. Water samples were centrifuged at 300 × *g* for 15 min, the supernatant was decanted, and a loopful of each pellet was suspended in 500 μl sterile distilled water and boiled for 30 min. PCR was performed by the protocol described previously, using *Mycobacterium*-specific forward primer 5′-GGTGGTTTGTCGCGTTGT TC-3 and reverse primer 5′-ATGCACCACCTGCACACAGG-3′) (20). The reaction mixtures contained 5-μl aliquots of the supernatants from water samples or 1 μl of soil DNA. Samples were considered "negative" if no 473-bp PCR amplification product (the predicted size) was observed on the gel. To check that the reaction was not inhibited in such cases, the PCR was repeated with *M. smegmatis* DNA in addition to the soil DNA.

Decontamination and culturing mycobacteria from soil and water. Several methods were compared, and the results were used to optimize the protocol described below, which proved to be suitable for isolating mycobacteria from the soil samples. A 2-g subsample of each composite soil sample was suspended in 5 ml nutrient broth (Oxoid) and incubated at 37°C for 2.5 h. For decontamination, a 2-ml aliquot was then centrifuged at 2,000 × *g* for 15 min. The pellet was resuspended in a solution containing 5 ml of malachite green in distilled H_2O (0.25 μg ml^{-1}), 1 ml of cycloheximide (500 μg ml^{-1}), and 5 ml of 4% NaOH and left to stand at room temperature for 20 min (6, 22). This preparation was neutralized by addition of 10 ml phosphate buffer (pH 6.9) and then centrifuged at 2,000 × *g* for 15 min; most of the supernatant was discarded, and the pellet was resuspended in the remaining 2.5 ml of supernatant. Aliquots (100 μl) of this suspension were inoculated in duplicate onto nutrient agar (Oxoid), selective nutrient agar containing cycloheximide (250 μg ml^{-1}) and nalidixic acid (35 μg ml^{-1}), and LJ medium slants (*Mycobacterium* Medium Manufacturers Ltd., Cardiff, United Kingdom) containing pyruvate and glycerol. All agar plates were sealed with Parafilm prior to incubation at either 28°C or 37°C. Cultures were examined after 3, 7, and 14 days and then every 2 weeks for up to 8 weeks.

Water cultures were obtained by centrifugation of samples at 2,000 × *g* for 15 min; the supernatant was decanted, and each pellet was suspended in the residual liquid and plated on media as described above.

The presence of mycobacteria was detected by Ziehl-Neelsen acid-fast staining (24) of smears from single colonies or from overgrown, mixed cultures. Many samples had to be discarded without further tests because of massive overgrowth by spore-forming fungi. Although some nonmycobacterial organisms are weakly acid fast as determined by ZN staining, the majority of strongly acid-fast, ZN stain-positive bacteria are assumed to be mycobacteria. When the ZN staining gave ambiguous results, DNA was extracted. A loopful of bacterial growth was suspended in 40 μl NaOH (0.25 M) and boiled for 30 s, and then 40 μl HCl (0.25 M), 20 μl Tris Cl (pH 8.0) (0.5 M), and 0.25% (wt/vol) Nonidet P-40 were added. The mixture was boiled 2 min, transferred to ice for 2 min, and centrifuged at 10,000 × *g* for 5 min, and then 1 μl of the supernatant was subjected to PCR with mycobacterium-specific primers as described above. Samples were considered "negative" if no acid-fast organisms were observed or no distinct PCR products could be amplified from microbial growth.

Some samples provided single colonies of acid-fast bacteria that could be maintained following subculture. DNA was extracted from these bacteria and amplified by PCR as described above for subsequent sequence analysis. However, many ZN stain-positive colonies could not be maintained as pure cultures and were subsequently discarded.

The statistical significance of differences in mycobacterial detection using either the culture or PCR method with soil samples obtained from the north and south taken in the wet or dry season was estimated using logistic regression.

TABLE 1. Samples from which 473-bp 16S rRNA gene fragments were sequenced in order to identify mycobacterial isolates cultured from soil and PCR fragments amplified from soil DNA

Village	Household	Sample	Site description	Concn (CFU g soil^{-1})	Soil PCR accession no.[a]	Isolate PCR accession no.[a]
Gweleweta, northern Karonga	1	S126	Open, mango trees	1×10^4	AJ420150 (9)	
	2	S128	Open, cattle, manure		AJ420161 (7)	
					AJ420152 (6)	
					AJ785777 (7)	AJ785766 (7)
	3	S132	Childrens' house		AJ420143 (32)	
					AJ420144 (4)	
	3	S133	Duck enclosure		AJ420147 (7)	AJ785773 (24)
					AJ420162 (7)	
	3	S134	Open, cattle enclosure		AJ785778 (13)	
					AJ420148 (26)	
					AJ420164 (17)	
	6	S160	Open, banana hedge	6×10^3	AJ420159 (29)	AJ785772 (24)
					AJ785779 (5)	
Kasyata, northern Karonga	1	S164	Bath enclosure, toilet		AJ420145 (37)	
	2	S175	Toilet, eucalyptus shade			AJ785771 (24)
	3	S188	Duck enclosure			AJ785770 (24)
	4	S8MJ	Toilet	1×10^4	AJ420151 (26)	
					AJ420149 (5)	
Kaporo, northern Karonga	Health center	S209	Lepra consultation, shade			AJ785774 (34)
Mwamtawale, southern Karonga	1	S58	Toilet	1×10^3	AJ420156 (20)	
	3	S93	Bath enclosure, mango tree	1×10^4	AJ420154 (20)	
Mzang' unya, southern Karonga	1	S64	Communal tap	1×10^3	AJ785780 (18)	
	2	S66	Open, bath, grass		AJ420157 (20)	
	2	S68	Communal tap		AJ420163 (10)	
	3	S72	Open, cultivated	2×10^2		AJ785775 (25)
Mgoyera, southern Karonga	1	S78	Open, rubbish dump	1×10^4	AJ785781 (31)	AJ785776 (20)
Chilumba, southern Karonga	Health center	S119a	Open, guardian shade	1×10^4	AJ785782 (2)	AJ785769 (24)
					AJ785783 (27)	
	KPS	S115	Woodlot		AJ420155 (20)	AJ785768 (24)
	KPS	CKPS	Chicken enclosure		AJ420153 (20)	
	KPS	GKPS	Garden			AJ765767 (24)
		Sp2525	Sputum culture		AJ420160 (7)	

[a] The numbers in parentheses are the branch numbers on the phylogenetic tree in Fig. 1.

Sequencing mycobacterial 16S rRNA. Fragments of the mycobacterial 16S rRNA gene amplified directly from soil or from isolates cultured from the soil samples were purified from agarose gels and cloned using TA cloning kits (Invitrogen). Plasmids were prepared using QIAGEN mini kits and were sequenced using ABI PRISM Big Dye terminator cycle sequencing Ready Reaction kits. The sequence analysis was performed using the GCG10 and PHYLIP programs.

Phylogenetic analysis. The 473-bp sequence amplified by the primers corresponds to positions 575 to 1048 in the 16S rRNA gene of *M. tuberculosis* (EMBL accession no. X58890) (16). Sequences amplified directly from soil or from cultured isolates were compared to the NCBI database using BLAST. All of the sequences were identical to or most closely related to sequences of *Mycobacterium* species. Using CLUSTALW, these sequences, together with the corresponding 473-bp sequences from other relevant mycobacteria (chosen because they have been implicated in disease or are commonly isolated from soil) were aligned; any sequences containing unknown or ambiguous bases were discarded. The corresponding sequence from the acid-fast actinomycete *Nocardia asteroides* was included as an outgroup. The alignment contained 51 bases that were different in different sequences, and 350 conserved bases were deleted from the alignment (52 conserved bases remained) to improve the resolution of the subsequent phylogenetic analysis using PHYLIP, version 3.6 (J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle, 2004). The maximum-likelihood method DNAML was used to produce a tree, and bootstrap values were estimated using SEQBOOT.

RESULTS

Phylogenetic analysis of amplified sequences. The 473-bp region of the 16S rRNA gene chosen for this study was variable in environmental isolates of mycobacteria (Table 1 and Fig. 1) and many rapidly growing species related to the *M. fortuitum* group, including some known human opportunistic pathogens (4). However, it cannot be used to discriminate in silico between many species that cause important human and animal diseases. Thus, *M. tuberculosis*, the *M. avium* complex (including *M. intracellulare* and *M. scrofulaceum*), *M. bovis*, *M. kansasii*, *M. marinum*, and *M. ulcerans* occur at the same position on the phylogenetic tree based on this region, although *M. leprae* is distinctly different (Fig. 1). The most similar environmental sequence obtained from soil DNA was from northern Karonga (sample S164, sequence accession no. AJ420145). This sequence is identical to the corresponding sequence of *M. tokaiense*, a species isolated from soil in Tokyo, Japan (25), and similar to sequences of two species that degrade polyaromatic hydrocar-

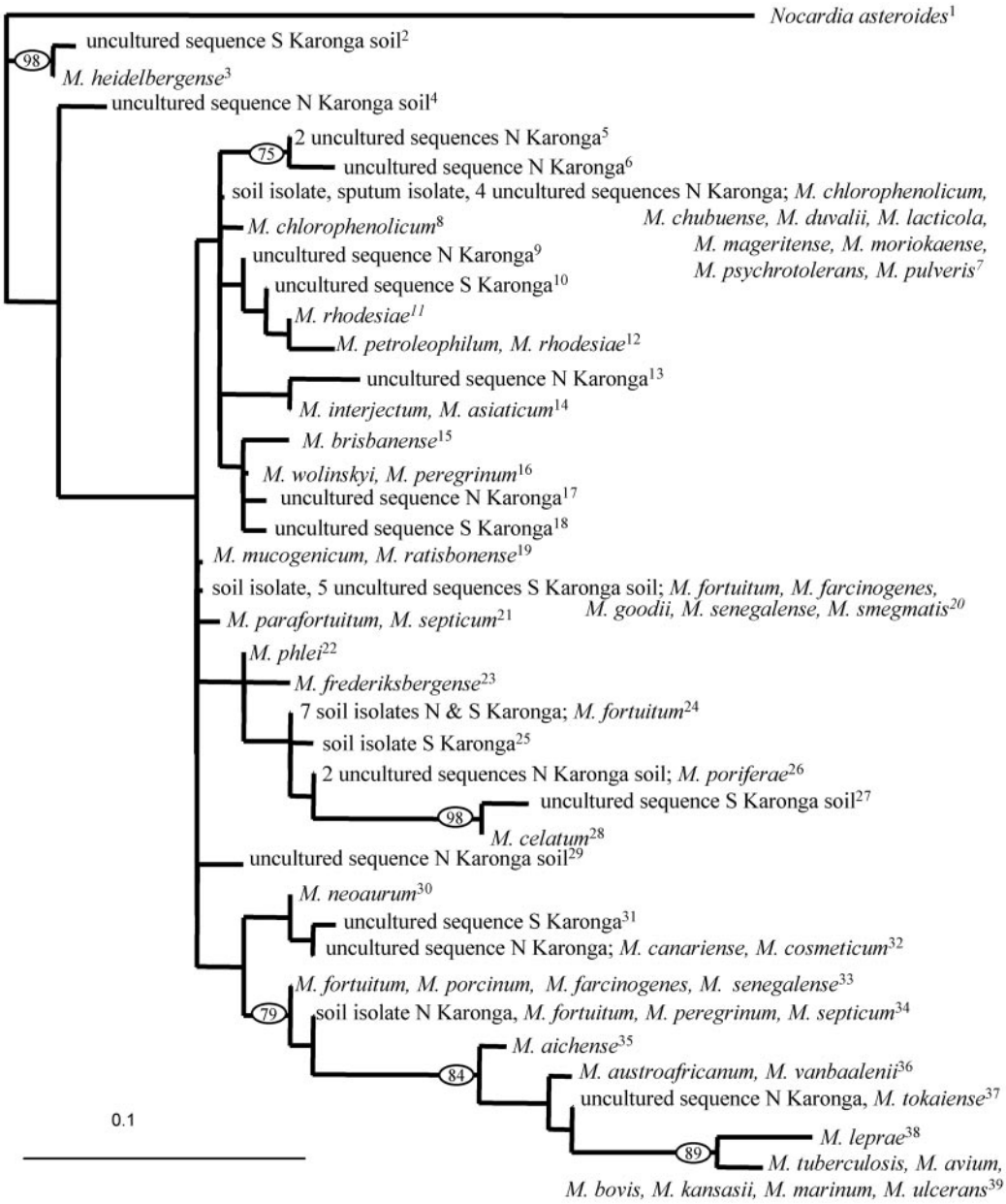

FIG. 1. Phylogenetic tree showing relationships between environmental sequences and isolates from Karonga District and known mycobacterial species. The tree is based on a comparison of a 473-bp sequence from the mycobacterial 16S rRNA gene, using maximum likelihood. Bootstrap values greater than 70% are indicated at the nodes. Bar = 0.1 substitution per site. The superscript numbers are branch numbers. Branch 1 contained the outgroup *Nocardia asteroides* strain DSM 43255 (accession no. AF430026). Branch 2 contained a sequence amplified from soil sample S119 from Chilumba in southern Karonga (AJ785782). Branch 3 contained *M. heidelbergense* ITG 7971 (AJ422048) and *Mycobacterium* sp. strain IWGMT 90174 (X88908). Branch 4 contained a sequence amplified from soil sample S132 from site 3A in Gweleweta in northern Karonga (AJ420144). Branch 5 contained sequences amplified from soil sample 8MJ from Kasyata (AJ420149) and soil sample S160 from Gweleweta (AJ785779) in northern Karonga. Branch 6 contained a sequence amplified from soil sample S128 from Gweleweta in northern Karonga (AJ420152). Branch 7 contained a cultured isolate from soil sample S132 from Gweleweta in northern Karonga (AJ785766), isolate 2525 from sputum obtained in Chilumba in southern Karonga (AJ420160), sequences amplified from soil samples S128, S132, and S133 from Gweleweta in northern Karonga (AJ420161, AJ785777, AJ420147, and AJ420162), *M. chlorophenolicum* PCP-1 (X79094), *M. chubuense* ATCC 27278 (AF480597), *M. duvalii* ATCC 43910 (U94745), *M. lacticola* ATCC 9626 (AF480582), *M. mageritense* DSM 44476 (AJ699399) and 1336 (AJ011335), *M. moriokaense* DSM 44221^T (AJ429044), *M. psychrotolerans* (AJ534886), *M. pulveris* DSM 44222^T (AJ429046), and *Mycobacterium* spp. strains SM7.6.1 (AF247497), WF2 (U90877), DSM 3803 (AY147261), HE5 (AJ012738), and PX3 (AY337605). Branch 8 contained *M. chlorophenolicum* DSM 43826 (X79292). Branch 9 contained a sequence amplified from soil sample S126 from Gweleweta in northern Karonga (AJ420150). Branch 10 contained a sequence amplified from soil sample S68 from Mzang'unya in southern Karonga (AJ420163). Branch 11 contained *M. rhodesiae* JS60 (AF498650). Branch 12 contained *M. petroleophilum* ATCC 21497 (AF480587), *M. rhodesiae* DSM44223^T (AJ429047), and *Mycobacterium* spp. strains PAH135 (X84978), GP1 (AJ012626), RJG11 (U30661), and TA27 (AB028482). Branch 13 contained a sequence amplified from soil sample S134 from Gweleweta in northern Karonga (AJ785778). Branch 14 contained *M. interjectum* MB739 (AJ272088), *M. asiaticum* ATCC 25276 (AF480595), and *Mycobacterium* spp. strains IWGMT 90100 (X88915), IWGMT90093 (X88915), and IWGMT 90203

bons, *M. austroafricanum* and *M. vanbaalenii* (17), and the vinyl chloride degrader *M. aichiense* (27). An uncultured sequence from sample S119a (AJ785782) from southern Karonga was most similar to (although distinct from) the sequence of another pathogenic mycobacterium, *M. heidelbergense* (11). In general, the uncultured sequences obtained from soil DNA fell into a few closely related groups; each sequence was similar to sequences of known *Mycobacterium* species, many of which had been isolated from soil at various locations worldwide, or was closely related to *M. fortuitum*. The shortest branch lengths on the tree represent single nucleotide differences; branch lengths indicate the degree of relatedness. The bootstrap values for most of the branches on the tree were less than 50%, indicating that many of the positions are interchangeable.

There was no clear difference between the pattern of sequences obtained from sites in the northern part of Karonga District and pattern of sequences obtained from sites in the southern part of Karonga District. The sources of 16S rRNA sequences amplified directly from soil DNA and of cultured isolates are shown in Table 1. Some of the uncultured sequences were found in soil from several sites, and in two cases the same (uncultured) sequences were also found in cultured isolates from soil. Identical sequences (Fig. 1, branch 7) were found in samples obtained from three different sites in Gweleweta in northern Karonga (samples S128, S132, and S133), a culture isolated from sample S132, and a culture isolated from sputum obtained from the KPS laboratories (Sp2525). These sequences were the same as the corresponding sequences from a group of species that included *M. chlorophenolicum*, which was first isolated from Finnish soil (12), *M. mageritense*, which was isolated from sputum in Spain (5), and *M. pulveris*, which was isolated from dust in Japan (26). Another group of sequences (Fig. 1, branch 20) included sequences obtained from a soil isolate from sites in southern Karonga (from sample S78) and also five uncultured soil DNA samples (samples S58, S93, S66, S115, and CKPS). These sequences were identical to the corresponding sequences in *M. fortuitum* strain ATCC 49403 and species associated with human and animal infections, including *M. smegmatis*, *M. goodii*, *M. farcinogenes*, and *M. senegalense* (1). Four isolates from northern Karonga samples (samples S133, S160, S175, and S188) and three isolates from southern Karonga samples (samples S119, S115, and GKPS) had sequences that were identical to the *M. fortuitum* strain DSM46621 sequence (Fig. 1, branch 24). Other sequences obtained from these isolates using primers FD1 and RD1 (to amplify most of the 16S rRNA gene) were also identical to the strain DSM46621 sequence, indicating that the isolates are probably closely related strains of *M. fortuitum.* Another southern Karonga soil isolate (from sample S72) differed by only 1 base in the 473-bp region, indicating that it is also closely related to DSM46621. Similarly, a soil isolate from northern Karonga sample S209 was identical in the 473-bp region to a group of mycobacterial species that included *M. fortuitum* MB44. It is interesting that in contrast to the most common class of soil isolates, no identical uncultured sequences were detected in soil DNA from northern and

(X88910). Branch 15 contained *M. brisbanense* ATCC 49938 (AY012577). Branch 16 contained *M. wolinskyi* ATCC 700009 (Y12871) and *M. peregrinum* ATCC 14467 (AF058712). Branch 17 contained a sequence amplified from soil sample S134 from Gweleweta in northern Karonga (AJ420164). Branch 18 contained a sequence amplified from soil sample S64 from Mzang'unya in southern Karonga (AJ785780). Branch 19 contained *M. mucogenicum* strains ATCC 49650 (AF480586) and ATCC 49649 (AF480585), *M. ratisbonense* (AF055331), SD4 (AJ271863), and *Mycobacterium* spp. soil isolates EM3 (AY436799) and BPC5 (AY162040). Branch 20 contained a cultured isolate from soil sample S78 from Mgyoyera in southern Karonga (AJ785776), sequences amplified from soil samples GKPS and S115 from Chilumba (AJ420153 and AJ420155), from samples S58 and S93 from Mwamtawale (AJ420156, AJ420154), and from sample S66 from Mzang'unya (AJ420157) in southern Karonga, *M. fortuitum* strain ATCC 49403 (AF480580), *M. smegmatis* strain DSM 43756 (AJ536041), *M. farcinogenes* strain NCTC 10955^T (AY457084), *M. goodii* strain M069 (Y12872), *M. senegalense* CIP 104941^T (AY457081), and United Kingdom soil isolate *Mycobacterium* sp. CF1 (AJ509013). Branch 21 contained *M. parafortuitum* DSM 43528 (X93183) and *M. septicum* MB804 (AJ416909). Branch 22 contained *M. phlei* ATCC 11758 (AF480603). Branch 23 contained *M. frederiksbergense* DSM 44346 (AJ276274). Branch 24 contained cultured isolates from soil samples GKPS, S115, and S119 from Chilumba in southern Karonga (AJ785767, AJ785768, and AJ785769) and from samples S133 and S160 from Gweleweta (AJ785773 and AJ785772) and samples S175 and S188 from Kasyata (AJ785771 and AJ785770) in northern Karonga, and *M. fortuitum* CIP 104534 (AY457066). Branch 25 contained a cultured isolate from soil sample S72 from Mzang'unya in southern Karonga (AJ785775). Branch 26 contained sequences amplified from soil sample S134 from Gweleweta (AJ420148) and sample 8MJ from Kasyata (AJ420151) in northern Karonga, *M. poriferae* ATCC 35087 (AF480589), and soil isolate *Mycobacterium* sp. strain JS621 (AF498659). Branch 27 contained a sequence amplified from soil sample S119 from Chilumba in southern Karonga (AJ785783). Branch 28 contained *M. celatum* DSM 44243 (AJ536040), ATCC 51130 (L08170), and ATCC 51131 (L08169). Branch 29 contained a sequence amplified from soil sample S160 from Gweleweta in northern Karonga (AJ420159). Branch 30 contained *M. neoaurum* ATCC 25795 (AF480593). Branch 31 contained a sequence amplified from soil sample S78 from Mgoyera in southern Karonga (AJ785781). Branch 32 contained a sequence amplified from soil sample S132 from Gweleweta in northern Karonga (AJ420143), *M. canariense* 502329 (AY255478), and *M. cosmeticum* ATCC BAA-878 (AY449728). Branch 33 contained *M. fortuitum* ATCC 49404 (AF480581), *M. boenickei* ATCC 49935 (AY012573), *M. farcinogenes* ATCC 35753 (AF055333), *M. houstonense* ATCC 49403 (AY012579), *M. neworleansense* ATCC 49404 (AY012575), *M. porcinum* ATCC 33776 (AF480588), and *M. senegalense* ATCC 35796 (AF480596). Branch 34 contained a cultured isolate from soil sample S209 from Kaporo in northern Karonga (AJ785774), *M. fortuitum* MB44 (AJ416910), *M. concordense* ATCC BAA-329 (AY012576), *M. peregrinum* ATCC 14467 (AJ42246), *M. septicum* HXN1900 (AJ457055), HXN500 (AJ457055), and W4964 (AF111809), *Mycobacterium* sp. strain NB01 (AY188086), and *Mycobacterium* sp. strain DhA-55 (AJ011510). Branch 35 contained *M. aichiense* JS618 (AF498656). Branch 36 contained *M. austroafricanum* ATCC 33464 (X93182) and *M. vanbaalenii* DSM 7251 (AY636003). Branch 37 contained a sequence amplified from soil sample S164 from Kasyata in northern Karonga (AJ420145), *M. tokaiense* ATCC 27282 (AF480590), and *Mycobacterium* spp. strains MA-112/96 (Y08857) and PP1 (AB009578). Branch 38 contained *M. leprae* TN (AL583920). Branch 39 contained *M. tuberculosis* CDC 1551 (AE007009), *M. avium* NIPHL070703A (AY360329), *M. avium* subsp. *paratuberculosis* k10 (AE017236), *M. africanum* ATCC 25420 (AF480605), *M. bovis* NIPHL 110703 (AY360331), *M. bohemicum* E743 (AJ277284), *M. gastri* ATCC 15754 (AF480602), *M. intracellulare* MB971 (AJ422047), *M. intermedium* (X67847), *M. kansasii* ATCC 12478 (AF480601), *M. liflandii* (AY500838), *M. marinum* ATCC 927 (AF456240), *M. microti* ATCC 19422 (AF480584), *M. pinnipedi* (AF502574), *M. scrofulaceum* ATCC 19981 (AF480604), and *M. ulcerans* Borstel 10564/70 (X58954).

TABLE 2. Numbers and proportions of soil and water samples from which mycobacteria were detected using the decontamination-culture-ZN staining method and/or the PCR method

Samples	Acid-fast culture result	No. (%) as determined by PCR		
		Positive	Negative	Total
Soil	Positive	54 (36)	22 (15)	76 (51)
	Negative	19 (13)	53 (36)	72 (49)
	Total	73 (49)	75 (51)	148 (100)
Water	Positive	12 (50)	6 (25)	18 (75)
	Negative	1 (4)	5 (21)	6 (25)
	Total	13 (54)	11 (46)	24 (100)

TABLE 3. Comparison of culture- and PCR-positive samples detected in soil samples from the north and south collected in the dry and wet seasons

Method	Season	No. positive/total no. (%)		
		North	South	Total
Culture	Dry	39/66 (59)	24/47 (51)	63/113 (56)
	Wet	6/19 (32)	7/16 (44)	13/35 (37)
	Total	45/85 (53)	31/63 (49)	76/148 (51)
PCR	Dry	44/66 (70)	21/47 (45)	65/113 (58)
	Wet	2/19 (11)	4/16 (25)	6/35 (17)
	Total	46/85 (54)	25/63 (40)	71/148 (48)

southern Karonga, possibly indicating that there were differences in the soil mycobacterial populations present.

The similarity of isolates cultured from different soil samples from northern and southern Karonga indicated that there was a potential bias in the decontamination and culture methods that may have favored the *M. fortuitum* group. Nevertheless, it is reassuring that in two cases identical sequences were found in both cultured isolates and uncultured soil DNA. Although the groups of multiple identical sequences from different sites did not include samples from both northern and southern Karonga (Fig. 1, branches 5, 7, 20, and 26), such groups were found in clusters of closely related sequences (Fig. 1, branches 9 and 10, 17 and 18, 26 and 27, and 31 and 32).

Comparison of mycobacterial detection by the decontamination-culture method and mycobacterial detection by the PCR method. As shown in Table 2, mycobacteria were detected in the majority of samples using either the culture or PCR method (64% in soil, 79% in water). The detection rates were similar for the two methods with the 148 soil samples (51% positive with the culture method, 49% positive with the PCR method), although only 36% were positive with both methods. For the 24 water samples, 75% of the samples were positive with the culture method and 54% were positive with the PCR method; 50% were positive with both methods.

Results by area and season. Table 3 shows the results for soil samples broken down by area (north versus south) and season (dry versus wet) for the culture method and the PCR method. The proportion of samples positive for mycobacteria was consistently higher in the dry season than in the wet season for both the culture ($P = 0.06$) and PCR ($P < 0.001$) methods. There was no convincing overall north-south difference, although with both methods the proportion of samples that were positive was higher in the north in the dry season and in the south in the wet season, and the differences were statistically significant for the PCR method ($P = 0.05$).

The results for the subset of 32 samples taken from the same sites in the north and south in the dry season and again in the wet season showed the same trends, with higher levels of recovery in the dry season (66%) than in the wet season (34%) when either method was used. No mycobacteria were detected in water samples taken in the wet season, although 95% of the samples taken in the dry season were positive with either method.

There was no obvious pattern of sites where mycobacteria were detected, nor was there any clear effect of soil pH (Table 4). Where the numbers of acid-fast bacteria could be determined, the population sizes could be estimated, and they ranged from 250 to 12,500 CFU g soil^{-1} (Table 4). However, in most cases the presence of other bacteria and fungi on the selective plates made estimation inaccurate or impossible; hence, only the presence or absence of mycobacteria was recorded.

DISCUSSION

This investigation showed that environmental mycobacteria are common in soil and water in Karonga District and that they are recovered more easily in the dry season than in the wet season. There was some evidence for regional differences (higher level of recovery from soil in the north than in the south in the dry season, but higher level of recovery in the south than in the north in the wet season). This is at least consistent with geographic differences, although the implications of species patterns remain unclear.

Because PCR can in theory detect mycobacteria whether they are actively growing, moribund, or dead, the slightly greater detection rates obtained by using culture methods than by using PCR were surprising. It is possible that some culture samples contained acid-fast bacteria that were not mycobacteria, but it is more likely that the soil DNA extract contained substances that inhibited the PCR that made the assay suboptimal. Quantification was difficult in most cases, but when the culture method resulted in discrete acid-fast colonies, the maximum population size detected was around 10^4 CFU per g soil, compared with the 10^6 to 10^8 culturable bacteria per g typically present in soil. The total population of these microorganisms is likely to fluctuate in the different seasons; many organisms probably die or cease to grow in harsh dry conditions, and the numbers may increase in the wet season. It is possible that the waxy cell wall of environmental mycobacteria confers a survival advantage in hot, dry soil but that these organisms are outcompeted by other microbial groups or grazed by soil predators when it is cooler and wetter. Also, mycobacteria may be more difficult to detect in the wet season, especially when PCR is used, because of increases in inhibitory microbial and organic components in the soil. Alternatively, EM may be washed out of the soil and diluted in water by

TABLE 4. Detection of mycobacteria using the culture-ZN staining method or the PCR method in soil samples collected from the same sites in the dry and wet seasons

Village	Household	Sample	Site description	Analysis results				Concn (CFU g soil^{-1})	Soil pH[a]
				Dry season		Wet season			
				ZN	PCR	ZN	PCR		
Kasyata, northern Karonga	1	S162	Open, brick storage	+	+	−	−	2×10^2	7.1
	1	S164	Bath enclosure, toilet	+	+	+	+		7.5
	1	S168	Palm tree plot	+	−	−	−	6×10^3	
	1	S169	Communal tap	−	−	−	−		5.6
	2	S174	Children's house	+	+	−	−	5×10^2	6.6
	2	S176	Bath enclosure, cashew	+	+	+	−		5.0
	2	S177	Crop processing	+	+	−	−		6.0
	3	S188	Duck enclosure	+	−	+	−		
	3	S190	Bath enclosure, cassava	−	−	−	−		
	3	S191	Toilet	+	+	+	−		4.8
	3	S192	Communal tap	−	+	+	−		4.5
	4	S197	Bath enclosure	+	+	−	−		
	4	S199	Well	+	+	−	−		
	4	S5MJ	Kitchen	+	+	+	+		
	4	S8MJ	Toilet	+	+	−	−	1×10^4	5.2
	5	S202	Toilet	−	+	−	−		5.0
	5	S203	Open space	−	−	−	−		7.3
	5	S206	Communal tap	−	+	−	−		6.1
Mwamtawale, southern Karonga	1	S54	Open, cultivated	−	−	+	+		
	1	S57	Bath enclosure	+	−	−	−		
	2	S80	Kitchen, bath enclosure	−	−	−	−		7.2
	2	S81	Toilet	−	−	+	−		6.0
	2	S82	Hedge	−	−	+	−		8.1
	2	S86	Borehole	+	+	−	−		6.9
	2	S88	Toilet	−	−	−	−		4.6
	3	S92	Kitchen	−	−	+	+		3.9
	3	S93	Bath enclosure, mango	+	−	−	−	1×10^4	6.9
	3	S94	Mango tree shade	−	−	−	−		
Mzang' unya, southern Karonga	2	S68	Communal tap	+	+	−	−		
	3	S69	Open, uncultivated	−	−	+	−		6.9
	3	S71	Kitchen, bath enclosure	−	+	−	−		7.2
	3	S73	Toilet	+	+	−	−		5.7

[a] Soil pH determined at the time of collection in the dry season.

heavy rain. The northern part of Karonga District has appreciably higher rainfall than the southern part, which is likely to be relevant to this issue.

The isolates cultured from soil collected in Mgoyera in southern Karonga (sample S78) and Gweleweta in northern Karonga (sample S160) had previously been identified as *M. fortuitum* using conventional biochemical tests. The ability of these isolates to block the *M. bovis* BCG vaccine in mice was compared to the ability of two strains belonging to the *M. avium* complex that were isolated from sputum collected from suspected TB patients in Karonga District (3). The study revealed no effect of prior exposure to the *M. fortuitum* isolates on the response to subsequent BCG challenge, whereas prior exposure to both *M. avium* complex isolates reduced the ability of BCG bacilli to grow, potentially compromising the efficacy of the vaccine. The different responses may have been due to the inability of the *M. fortuitum* isolates to grow in the mice, in contrast to *M. avium* complex strains, as well as to differences in surface antigens between the two groups of EM. No sequences corresponding to the group that includes the *M. avium* complex and other human and animal pathogens were detected in soil or water during this study, although there have been many reports that such sequences have been isolated from various environmental samples. Recent research has shown that *M. avium* subsp. *paratuberculosis* introduced into soil in fecal material remains detectable for up to 55 weeks in the United Kingdom, depending on the soil and shade conditions (28). It is possible that *M. avium* and other mycobacteria implicated in disease are scarce in soils in Karonga District and thus were not detectable against the background of more common environmental mycobacteria. It would be interesting to see if more specific primers could detect infrequent mycobacteria or if different (e.g., two-stage) decontamination methods would allow culture of such organisms. Alternatively, people in Karonga and other rural areas of Africa may be exposed to *M. avium* and similar species by contact with animals, birds, insects, or some other environmental source that was not investigated in this study. For example, aquatic insects and plants act as a reservoir for the causative organism of Buruli ulcer, *M. ulcerans* (18, 19).

Although more samples collected in the northern part of Karonga District than in the southern part were positive for mycobacteria, particularly in the dry season, there was no obvious pattern of distribution; the majority of environmental

isolates and uncultured DNA sequences resembled one another and appeared to be related to known nonpathogenic species. Some of the Karonga isolates and sequences may represent species which have never been fully characterized. Indeed, the variety of the environmental flora presents an immense challenge, as many of the organisms likely to have direct effects on soil quality and agriculture, as well as indirect effects on human health, have yet to be described. The work presented here, carried out in the context of epidemiological studies of human disease, provides a glimpse of this complexity. There were minor 16S rRNA sequence differences in isolates from the northern and southern parts of Karonga District, but overall the EM from the two regions, whether cultured or identified by PCR directly from soil, appear to be similar. Although this investigation revealed no clear explanation for the different responses of humans to mycobacterial antigens between the northern and southern parts of Karonga District, it showed that the season in which samples are collected has a strong influence on the detection rate. This may be an important consideration in future sampling of EM in Malawi and elsewhere.

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REFERENCES

1. **Adekambi, T., and M. Drancourt.** 2004. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp*65, *sodA*, *recA* and *rpoB* gene sequencing. Int. J. Syst. Evol. Microbiol. **54:**2095–2105.
2. **Black, G. F., R. E. Weir, S. D. Chaguluka, D. Warndorff, A. C. Crampin, L. Mwaungulu, L. Sichali, S. Floyd, L. Bliss, E. Jarman, L. Donovan, P. Andersen, W. Britton, G. Hewinson, K. Huygen, J. Paulsen, M. Singh, R. Prestidge, P. E. Fine, and H. M. Dockrell.** 2003. Gamma interferon responses induced by a panel of recombinant and purified mycobacterial antigens in healthy, non-*Mycobacterium bovis* BCG-vaccinated Malawian young adults. Clin. Diagn. Lab. Immunol. **10:**602–611.
3. **Brandt, L., J. Feino Cunha, A. Weinreich Olsen, B. Chilima, P. Hirsch, R. Appelberg, and P. Andersen.** 2002. Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. Infect. Immun. **70:**672–678.
4. **Brown-Elliot, B. A., and R. J. Wallace, Jr.** 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin. Microbiol. Rev. **15:**716–746.
5. **Domenech, P., M. S. Jimenez, M. C. Menendez, T. J. Bull, S. Samper, A. Manrique, and M. J. Garcia.** 1997. *Mycobacterium mageritense* sp. nov. Int. J. Syst. Bacteriol. **47:**535–540.
6. **Donoghue, H. D., E. Overend, and J. L. Stanford.** 1997. A longitudinal study of environmental mycobacteria on a farm in South-West England. J. Appl. Microbiol. **82:**57–67.
7. **Falkinham, J. O., III, C. D. Norton, and M. W. LeChevallier.** 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol. **67:**1225–1231.
8. **Fine, P. E. M.** 1995. Variation in protection by BCG: implications of and for heterologous immunity. Lancet **346:**1339–1345.
9. **Fine, P. E. M., J. A. C. Sterne, J. M. Pönnighaus, and R. J. W. Rees.** 1994. Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity. Lancet **344:**1245–1249.
10. **Fine, P. E., S. Floyd, J. L. Stanford, P. Nkhosa, A. Kasunga, S. Chaguluka, D. K. Warndorff, P. A. Jenkins, M. Yates, and J. M. Pönnighaus.** 2001. Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. Epidemiol. Infect. **126:**379–387.
11. **Haas, W., W. R. Butler, P. Kirschner, B. B. Plikaytis, M. B. Coyle, B. Amthor, A. G. Steigerwalt, D. J. Brenner, M. Salfinger, J. T. Crawford, E. C. Bottger, and H. J. Bremer.** 1997. A new agent of mycobacterial lymphadenitis in children: *Mycobacterium heidelbergense* sp. nov. J. Clin. Microbiol. **35:**3203–3209.
12. **Häggblom, M. M., L. J. Nohynek, N. J. Palleroni, K. Kronqvist, E. L. Nurmiaho-Lassila, M. S. Salkinoja-Salonen, S. Klatte, and R. M. Kroppenstedt.** 1994. Transfer of polychlorophenol-degrading *Rhodococcus chlorophenolicus* (Apajalahti et al. 1986) to the genus *Mycobacterium* as *Mycobacterium chlorophenolicum* comb. nov. Int. J. Syst. Bacteriol. **44:**485–493.
13. **Kamala, T., C. N. Paramasivan, D. Herbert, P. Venkatesan, and R. Prabhakar.** 1994. Evaluation of procedures for isolation of nontuberculous mycobacteria from soil and water. Appl. Environ. Microbiol. **60:**1021–1024.
14. **Kamala, T., C. N. Paramasivan, D. Herbert, P. Venkatesan, and R. Prabhakar.** 1994. Isolation and identification of environmental mycobacteria in the *M. bovis*-BCG trial area of South India. Appl. Environ. Microbiol. **60:**2180–2183.
15. **Karonga Prevention Trial Group.** 1996. Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed *Mycobacterium leprae* vaccine for prevention of leprosy and tuberculosis in Malawi. Lancet **348:**17–24.
16. **Kempsell, K. E., Y. E. Ji, I. C. Estrada, M. J. Colston, and R. A. Cox.** 1992. The nucleotide sequence of the promoter, 16S rRNA and spacer region of the ribosomal RNA operon of *Mycobacterium tuberculosis* and comparison with *Mycobacterium leprae* precursor rRNA. J. Gen. Microbiol. **138:**1717–1727.
17. **Khan, A. A., S. J. Kim, D. D. Paine, and C. E. Cerniglia.** 2002. Classification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Mycobacterium* sp. strain PYR-1, as *Mycobacterium vanbaalenii* sp. nov. Int. J. Syst. Evol. Microbiol. **52:**1997–2002.
18. **Marsollier, L., T. Stinear, J. Aubry, J. P. Saint Andre, R. Robert, P. Legras, A.-L. Manceau, C. Audrain, S. Bourdon, H. Kouakou, and B. Carbonnelle.** 2004. Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. Appl. Environ. Microbiol. **70:**1097–1103.
19. **Marsollier, L., R. Robert, J. Aubry, J. P. Saint Andre, H. Kouakou, P. Legras, A.-L. Manceau, C. Mahaza, and B. Carbonnelle.** 2002. Aquatic insects as a vector for *Mycobacterium ulcerans*. Appl. Environ Microbiol. **68:**4623–4628.
20. **Mendum, T. A., B. Z. Chilima, and P. R. Hirsch.** 2000. The PCR amplification of non-tuberculous mycobacterial 16S rRNA sequences from soil. FEMS Microbiol. Lett. **185:**189–192.
21. **Pönnighaus, J. M., P. E. M. Fine, J. A. C. Sterne, R. J. Wilson, E. Msosa, P. J. K. Gruer, P. A. Jenkins, S. B. Lucas, N. G. Liomba, and L. Bliss.** 1992. Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. Lancet **339:**636–639.
22. **Portaels, F., A. De Muynck, and M. P. Sylla.** 1988. Selective isolation of mycobacteria from soil: a statistical analysis approach. J. Gen. Microbiol. **134:**849–855.
23. **Primm, T. P., C. A. Lucero, and J. O. Falkinham III.** 2004. Health impacts of environmental mycobacteria. Clin. Microbiol. Rev. **17:**98–106.
24. **Shanson, D. C.** 1982. Microbiology in clinical practice. Wright, Sevenoaks, United Kingdom.
25. **Tsukamura, M., S. Mizuno, and S. Tsukamura.** 1981. Numerical analysis of rapidly growing scotochromogenic mycobacteria, including *Mycobacterium obusense* sp. nov., nom. rev., *Mycobacterium rhodesiae* sp. nov., nom. rev., *Mycobacterium aichiense* sp. nov., nom. rev., *Mycobacterium chubuense* sp. nov., nom. rev., and *Mycobacterium tokaiense* sp. nov., nom. rev. Int. J. Syst. Bacteriol. **31:**263–275.
26. **Tsukamura, M., S. Mizuno, and H. Toyama.** 1983. *Mycobacterium pulveris* sp. nov., a nonphotochromogenic mycobacterium with an intermediate growth rate. Int. J. Syst. Bacteriol. **33:**811–815.
27. **Tsukamura, M., H. J. Van Der Meulen, and W. O. K. Grabow.** 1983. Numerical taxonomy of rapidly growing, scotochromogenic mycobacteria of the *Mycobacterium parafortuitum* complex: *Mycobacterium austroafricanum* sp. nov. and *Mycobacterium diernhoferi* sp. nov., nom. rev. Int. J. Syst. Bacteriol. **33:**460–469.
28. **Whittington, R. J., D. J. Marshall, P. J. Nicholls, I. B. Marsh, and L. A. Reddacliff.** 2004. Survival and dormancy of *Myobacterium avium* subsp. *paratuberculosis* in the environment. Appl. Environ. Microbiol. **70:**2989–3004.