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Vice Provost and Dean
of Graduate Studies

Thesis
2003
.D26

ANALYSIS OF *MYCOBACTERIUM* SPECIES FOR THE PRESENCE OF A MACROLIDE TOXIN, MYCOLACTONE

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Alexandria Daniel
May 2003

ACKNOWLEDGEMENTS

I would like to thank everyone who has made this thesis possible. First of all I would like to thank Dr. Pamela L. C. Small for her wonderful leadership, guidance, and help throughout this period. Furthermore, I would like to thank the members of my committee, Dr. Patricia K. Tithof and Dr. Robert N. Moore, for reviewing this thesis. Also, I would like to thank Dr. Richard Lee for the mass spectroscopy data, Dr. Francoise Portaels for her donation of environmental strains, and Dr. Marguerite Butler for her help with the phylogenetic analysis. I would like to extend my gratitude to all the members of the Small lab, especially Armand, Brian, Terri, and Sarojini, for all of their help throughout this journey. Also I would like to give many thanks to my friends Marc Cook and Sarah Sullivan, for their patience, support, and love to help me through this process. Finally I would like to thank my loving parents and my two wonderful sisters for all they have done throughout the years, making all of this possible.

ABSTRACT

Mycobacterium ulcerans is an environmental organism which is responsible for the disease Buruli ulcer, a resistant, severe, necrotizing skin disease. Buruli ulcer occurs in West and Central Africa, Australia, and Asia, but it appears to be increasing significantly in West Africa. *M. ulcerans* produces a polyketide-derived macrolide, mycolactone, which is required for virulence. *Mycobacterium* is a large genera with diverse species. Most of these species are non-pathogens present in soil and water. Although mycolactone production has been demonstrated in *M. ulcerans* from many countries, a search for mycolactone in other *Mycobacterium* species has not been undertaken. We have extracted polyketides from 46 *Mycobacterium* isolates, analyzed them with thin-layer chromatography (TLC), and tested them in a murine fibroblast cell line (L929) to investigate whether mycolactone or a similar lipid are produced by *Mycobacterium* species other than *M. ulcerans*. In these studies acetone soluble lipids (ASL) were prepared from a chloroform-methanol extract obtained from 4 fast growing species and 25 slow growing species of *Mycobacterium*. Among the slow growing species were 16 uncharacterized, species of *Mycobacterium* isolated from aquatic sources in areas endemic to *M. ulcerans* infection. Biochemical and cell culture assays of these lipids showed that whereas several strains of mycobacteria studied produced toxic lipids when tested in cell culture, none made mycolactone. Whereas mycolactone is toxic at picogram amounts, none of the lipids tested from other *Mycobacterium* species were active at less than 10 μg amounts, and none produced a cellular phenotype consistent with mycolactone activity. Two species of *Mycobacterium*, *M. scrofulaceum* and *M. kansasii*, contained lipids cytopathic to L929 fibroblasts at a concentration of less than 20 μg/μl.

In addition, eight of the environmental isolates contained cytopathic lipids. Analysis of the 16S rRNA sequence of these 8 environmental isolates of mycobacteria with cytopathic ASLs suggest that these 8 environmental isolates are novel species of *Mycobacterium*. Results from these studies show that although production of cytopathic lipids is relatively common among *Mycobacterium* species, the production of mycolactone appears so far to be restricted to *M. ulcerans*.

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NOMENCLATURE

g	gram
h	hour
kg	kilogram
log	logarithm
M	molar
mM	millimolar
in.Hg	inches mercury
min	minute
ml	milliliter
m/z	mass to charge ratio
ng	nanogram
uv	ultraviolet
vol	volume
µg	microgram
µm	micrometer
wt/vol	weight per volume
ng/ml	nanogram per milliliter
µg/ml	microgram per milliliter
µg/µl	microgram per microliter

ABBREVIATIONS

ASL	acetone soluble lipid
ATCC	American Tissue Culture Collection
BCG	bacillus Calmette-Guerin
BLAST	Basic Local Alignment Search Tool
bp	base pair
CEI	Congo Environmental Isolate
CF	culture filtrate
CPA	cytopathic activity
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
E value	Expect value
HIV	human immunodeficiency virus
IS	insertion sequence
LB	Luria Bertani
LPS	lipopolysaccharide
MEGA	Molecular Evolutionary Genetic Analysis
MS	mass spectroscopy
NJ	neighbor joining
OADC	oleic acid, bovine serum albumin, dextrose, and catalase enrichment
PCR	polymerase chain reaction

PDIM	phthiocerol dimycocerosate
PGL-1	phenolic glycolipid-1
PKS	polyketide synthase
R_f	ratio to front
rRNA	ribosomal ribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
TLC	thin layer chromatography
TMC	Trudeau Mycobacterial Culture
TMCC	Trudeau Mycobacterial Culture Collection
U	unit
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

I. Buruli Ulcer Disease:

Mycobacterium ulcerans is the causative agent of Buruli ulcer, an emerging infection in West Africa. The first clinical description of Buruli ulcer was reported in Australia in 1937 and designated the "Bairnsdale ulcer," however, the ulcers were thought to be due to *Mycobacterium tuberculosis* or *Mycobacterium leprae* (1). It was not until 1948 that the association between the disease and *M. ulcerans* was established. However, Sir Albert Cook had described the disease, which had been well known in Africa before this in 1897 (35). It is now recognized that epidemics of Buruli ulcer have occurred for over 100 years and that *M. ulcerans* is endemic in much of West and Central Africa. Buruli ulcer is now the third most common mycobacterial disease in non-immunocompromised people behind tuberculosis and leprosy (37).

The disease is characterized by progressive, severe necrotizing skin lesions that do not respond to antimicrobial therapy and require surgical excision as treatment (37) (Figure 1). Most of these lesions occur on the extremities (14). The first stage of disease (preulcerative stage) is indicated by a papule or a non-tender nodule or plaques with large areas of indurated skin. At later stages these lesion may progress to a plaque or edematous form. Histopathology of Buruli ulcer shows necrosis of the subcutaneous fat and vascular damage, which results in sloughing and secondary ulceration of the overlying skin with undermined edges (37) (Figure 2). Massive clusters of extracellular



Figure 1. Ulcerative lesion of Buruli ulcer on the upper right arm of a male. Adapted from van der Werf, 1999.

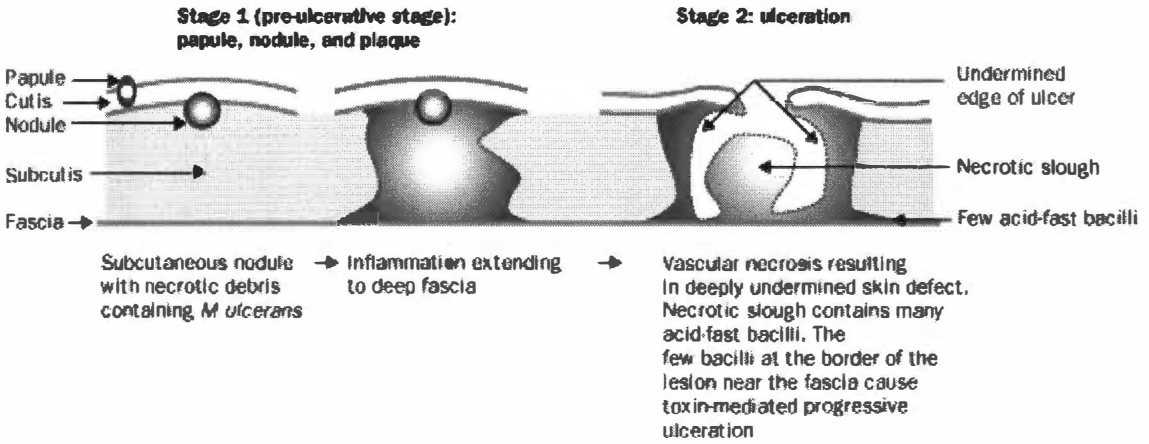


Figure 2. Stages of Buruli ulcer disease. From van der Werf, 1999.

acid-fast bacteria can be found within the necrotic tissue; however, the necrosis of the tissue spreads beyond these clusters of bacteria. The presence of tissue damage which extends beyond the presence of the organisms was the first clue that *M. ulcerans* might produce a secreted toxin (4). A distinct feature of Buruli ulcer is that organisms are found extracellularly, unlike all other mycobacterial infections, such as leprosy and tuberculosis, where organisms are found intracellularly (14, 35).

Buruli ulcer is a rural disease with wide distribution. Cases have been identified in Africa, Asia, South America, and low-lying marshy regions in Australia. Lesions clinically identical to those in humans have also been seen in koalas and ringtail possums in Australia (39). The disease has been described in more than 30 countries and an unexplained recent increase in the occurrence of the disease has occurred in West Africa (7).

M. ulcerans is an acid-fast, slow growing member of the environmental mycobacteria. Like *M. leprae* and *M. marinum, M. ulcerans* does not grow at 37°C and has an optimal growth temperature of 31-33°C (35). The mode of transmission of *M. ulcerans* is unknown. Epidemiological evidence defines proximity to slow-moving water as a previous risk factor for Buruli ulcer, and the bacterium cannot enter through intact skin (35). Recent evidence has implicated the involvement of aquatic insects as potential vectors in the infection process. Portaels et al. found several species of swamp insects that were PCR positive for *M. ulcerans*. Furthermore, they proposed that water-filtering organisms concentrate the bacteria and pass them on to aquatic predators, which pass the disease on to humans by biting (30). Recently, Marsollier et al. was able to infect

aquatic insects (Naucoridae) with *M. ulcerans* and transfer this infection to laboratory mice through biting. Furthermore, they were able to detect *M. ulcerans* in the salivary glands of these aquatic insects as well as others (23).

II. Identification of *M. ulcerans*:

Molecular diagnosis of *M. ulcerans* is possible through polymerase chain reaction (PCR) of two insertion sequences (ISs), IS2404 and IS2606, which have high specificity for *M. ulcerans*. IS elements do not have an essential function for the cell, but they do have the capability of promoting rearrangements, sequestering genes, and modifying gene expression. These IS elements are found in *M. ulcerans* in at over 50 copies each. Furthermore, these IS elements are nearly unique to *M. ulcerans*, with the exception of the identification of IS2606 in *Mycobacterium lentiflavum* (34). Previous diagnostic methods relied on staining the necrotic tissue samples from the lesion and identifying the presence of extracellular acid-fast bacilli (37). The use of these PCR probes as an identification for *M. ulcerans* is highly sensitive and specific.

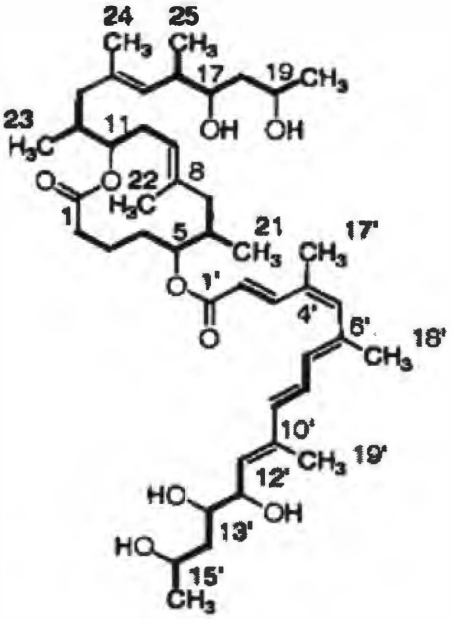
III. Polyketides:

Polyketides are biologically active lipid molecules which have important medicinal activities such as antibiotic, anticancer, antifungal, antiparasitic, and immunosuppressive properties (33). Polyketides are related by a common method of biosynthesis, not common structural features. Polyketide biosynthesis is very similar to fatty acids biosynthesis. In both cases synthesis is based upon repeated condensations of

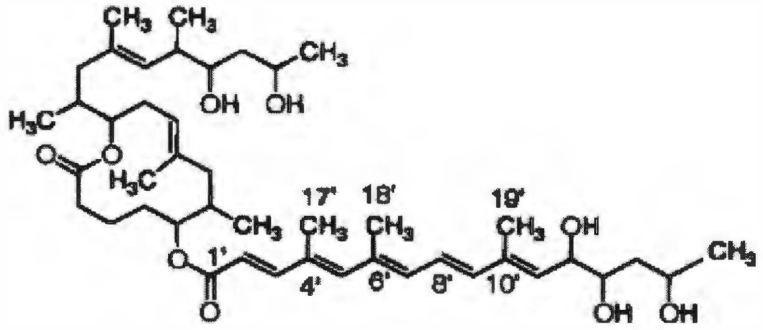
acyl groups onto a growing carbon chain. The enzymes responsible for polyketide synthesis, polyketide synthases (PKS), are classified into type I or type II based on how closely they mimic fatty acid synthases for vertebrates (type I) or for bacterial and plant (type II). However, polyketides are much more diverse than fatty acids because synthesis of a single molecule may utilize several different extender units. In addition, in some cases (type I PKSs) post-PKS modifications occur (27). Many structural subgroups of polyketides exist, and these include aromatics, macrolides, polyethers, and polyenes (38). Macrolides are formed from branched-chain fatty acids that cyclize to form a lactone ring. Erythromycin, produced by *Saccharopolyspora erythraea*, is a polyketide antibiotic belonging to the macrolide class (18). *M. ulcerans* produces a polyketide-derived macrolide, mycolactone, which is required for the organism's virulence (10).

IV. Mycolactone Toxin:

Mycolactone is a 12-membered macrolide ring formed by joining two polyketide chains to produce a hybrid polyketide. Mycolactone exists as a mixture of two isomeric compounds, mycolactone A and B which occur in about a 3:2 ratio, with mycolactone A being the major compound (13) (Figure 3). One of these two polyketide chains which form mycolactone A/B undergoes cyclization to form the 12-membered core lactone ring (8). Mycolactone is a relatively polar lipid and is obtained by chloroform-methanol (2:1, vol/vol) extraction of culture filtrate or bacteria. The partially purified extract is enriched for mycolactones by adding ice-cold acetone, a process which removes the phospholipids and leaves the acetone soluble lipids (ASL) (9).



1



2

Figure 3. Mycolactone isomers: mycolactone A, structure 1, and mycolactone B, structure 2. Adapted from Gunawardana, 1999.

Mycolactone exhibits a specific cytopathic effect on murine L929 fibroblasts, characterized by cell rounding within 24 hours, cell cycle arrest in the G_0/G_1 phase of the cell cycle by 36 hours, followed by apoptosis in 72 hours. Injection of guinea pigs with mycolactone produces a lesion with the same characteristics as *M. ulcerans* infection, namely, a painless, necrotic, non-inflammatory lesion, whereas, a mycolactone negative mutant of *M. ulcerans* fails to develop a lesion on guinea pigs (10). This evidence clearly indicates that mycolactone plays a major role in the development of these necrotic lesions seen with Buruli ulcer.

For *M. ulcerans*, Trudeau Mycobacterial Culture Number 1615, maximum cytopathic activity is associated with an ultraviolet (uυ)-active, light yellow component with a ratio to front value (R_f) of 0.23 in a solvent system of chloroform-methanol-water (90:10:1, vol/vol/vol) (10). It has recently been shown that *M. ulcerans* 1615 produces mycolactone congeners, minor species of mycolactone, in addition to mycolactone A/B (2). Also, it has recently been shown that mycolactones are four heterogeneous groups of macrolides which vary according to the geographic origin of the species of *M. ulcerans*. Mycolactone A/B is the major mycolactone component of ASLs for *M. ulcerans* 1615, a Malaysian isolate, as well as for African isolates (24). Mycolactone A/B is a cell associated lipid that produces a peak at *m/z* 765.5 $[M+Na]^+$ under mass spectroscopy (MS) analysis (10). MS analysis of strains from different regions shows that mycolactone A/B is also a component of Australian strains and Asian strains ASLs, but it is not the major component. Mycolactone C is the name given to the major species of the Austrailian isolates and it is indicated on MS as a peak at *m/z* 749.5. Asian species

contained a major peak at *m/z* 781.3 on MS analysis and this molecule is termed mycolactone D. Finally, neither mycolactone A/B, C, or D can be detected in Mexican strains although ASLs of *M. ulcerans* from Mexico exhibit typical mycolactone cytopathicity (24).

A lipid component of 1615 *M. ulcerans* ASLs, termed the core lactone, exhibits a cytopathic phenotype similar to that of mycolactone A/B on L929 cells. This core lactone is believed to be a precursor molecule for mycolactone. The core lactone is 10,000 times less active than mycolactone A/B. MS analysis of the core lactone shows it to be a peak at *m/z* 447.4 $[M+Na]^+$ (24).

Inflammatory cells are conspicuously lacking from Buruli ulcer lesions due to the presence of mycolactones. Several studies have shown that mycolactone is an immunosuppressive molecule which suppresses a T-cell mediated immune response as well as the acute inflammatory response. Antibody responses to the organism can be detected although antibodies to mycolactone are not (12). Unlike most environmental mycobacteria, the disease is not associated with infection with human immunodeficiency virus (HIV). There does not appear to be an increase in Buruli ulcer disease seen among patients with HIV. Furthermore, patients infected with both HIV and Buruli ulcer disease do not have clinical presentations that are different from non-immunocompromised patients (26).

V. Management of Disease:

Surgical excision of the lesion is so far the only effective means of treatment. Excision of all of the necrotic tissue as well as some of the healthy tissue is necessary, to prevent a reoccurring infection from residual *M. ulcerans*. Skin grafting is necessary for repair of larger lesions, and plastic surgery or physiotherapy may be needed for extensive lesions. In most cases treatment of the lesions with antimycobacterial agents have been ineffective. Drugs such as clofazimine, an antileprosy drug, rifampicin, streptomycin, and dapsone are all effective against *M. ulcerans in vitro,* but are not effective in combating these lesions (37). There is no current vaccine for the disease, although some evidence suggests that the bacillus Calmette-Guerin (BCG) vaccine may have a role in preventing the more serious forms of the disease (29).

VI. Lipid Virulence Determinants in *Mycobacterium* Species:

Lipids are important virulence determinants for many microorganisms. For example, the major virulence factor of Gram-negative bacteria is lipopolysaccharide (LPS). LPS, a major component of Gram-negative bacteria outer membranes, is a glycolipid which contains lipid A, the active structure moiety of LPS. Studies have shown that LPS is a major virulence factor of Gram-negative bacteria because purified LPS or chemically synthesized lipid A are able to reproduce some manifestations of septic shock in man and animals that is seen with Gram-negative bacterial infection (15). *Mycobacterium* species lack LPS as a virulence factor, but their cell walls contain many biologically active, complex lipid molecules. *M. tuberculosis* contains phthiocerol

dimycocerosate (PDIM), a cell-wall associated, polyketide derived lipid required for virulence, which appears to play a role in the growth of *M. tuberculosis* in the lungs. Mutants that have a disrupted synthesis of their PDIM are unable to colonize murine lungs (6). Another polyketide lipid which is a component of *M. leprae* cell wall, phenolic glycolipid-1 (PGL-1) has recently been implicated as a major factor in the neuropathogenesis of leprosy. Recent data suggests that PGL-1 may be involved in the demylination process which occurs in leprosy (31). Recently, evidence suggests that other *M. tuberculosis* polyketides play a role in the ability of *M. tuberculosis* to cause persistent infection (5).

Macrolides are a special category of polyketides that contain a lactone ring (18). Soil bacteria such as *Streptomyces* and *Saccharopolyspora* produce macrolides as secondary metabolites (19). However, mycolactone is the first known macrolide produced by a human pathogen and the only known macrolide to be produced by a *Mycobacterium* species (13). Genomic evidence from many *Mycobacterium* species has revealed that *Mycobacterium* species, like the *Streptomyces* species, contain many polyketide genes. None of these polyketide genes, however, appear capable of encoding a molecule as large as mycolactone. Although the *M. tuberculosis* genome suggests that *M. tuberculosis* could not produce a polyketide such as mycolactone, a systemic search for similar molecules in other *Mycobacterium* species has not been conducted (3). Mycolactone appears to be encoded by two polyketide enzymes, PKS Is, and we have recently shown that the core lactone encoded by PKS I has cytopathic activity though its potency is lower than that of mycolactone (24). Thus a *Mycobacterium* species that

contains only a polyketide synthase for the core lactone ring would still be active. In this study we have extracted lipids from a collection of *Mycobacterium* species and used cytopathic activity along with biochemical analysis to determine if these other mycobacteria make mycolactone or mycolactone related molecules.

VII. Objective:

Although clinical data strongly suggest that *M. ulcerans* is the only *Mycobacterium* species which produces a secreted toxin, studies have never been conducted on a large number of species of *Mycobacterium* to determine whether mycolactone or related molecules are present. Two recent findings led us to investigate the distribution of this molecule among *Mycobacterium* species. First we have shown that mycolactone is a hybrid polyketide comprised of two polyketides. We have shown that the product of the PKS gene, which encode the lactone core is active at high concentrations (24). The production of this molecule can be detected using tissue culture assays. Secondly, the presence of large numbers of IS elements in *M. ulcerans* raises the possibility that some or all of the genes encoding mycolactone may have been acquired by horizontal transfer from other bacteria (34). If these other bacteria lacked factors for colonization of the human host they would probably not have been detected. We were particularly interested in investigating whether environmental mycobacteria isolated from areas endemic for Buruli ulcer produced mycolactones.

In this study ASLs of 46 isolates from 29 different species of fast and slow growing mycobacteria were extracted and analyzed for the presence of mycolactones.

Included in this study are 16 slow growing, uncharacterized, environmental isolates of mycobacteria, which were isolated from water in regions endemic to *M. ulcerans* infection within the Democratic Republic of Congo. Although we found that several mycobacteria produce cytopathic lipids, the specific cytopathic phenotype produced was not similar to that of mycolactone. Of the mycobacteria from which cytopathic lipids were extracted, two pathogenic species *Mycobacterium scrofulaceum* and *Mycobacterium kansasii*, contained lipids that are cytopathic to L929 fibroblasts at a concentration of less than 20 μg/μl. Also, it was surprising to find that 8 of the Congo environmental isolates (CEI) also produced cytopathic polar lipids. None of these cytopathic lipids were as potent as mycolactone, and more importantly they all have a markedly different cytopathic effect on L929 fibroblasts. Furthermore, we tried to isolate these cytopathic lipids through the use of a chromatotron to determine if these cytopathic molecules could perhaps be due to a PKS for the core lactone. Two species of *Mycobacterium*, *M. tuberculosis* and *M. marinum*, are closely related to *M. ulcerans* (36). However, neither of these species were included in this study because previous studies have indicated that neither *M. tuberculosis* nor *M. marinum* produce mycolactones (3, 9).

Finally, in this study we further characterize the 8 CEI with cytopathic lipids. We have used 16S rRNA gene (16S rDNA) data to show that none of the CEI with cytopathic lipids are *M. ulcerans*. Kirschner et al. found that the 16S rRNA gene is an accurate and versatile method for identifying mycobacteria (21). The 16S rRNA gene is a valuable tool in determining phylogenetic relationships because it has both conserved

and variable regions. These highly conserved regions remain so due to structural and functional constraints on the molecule. However, the variable regions are species specific and can therefore be used for identification of species (32). Therefore, primers are designed within the conserved regions of the 16S rRNA gene and used to amplify the variable regions. We have used primers designed by Hughes et al. to amplify two variable regions within mycobacteria 16S rRNA gene (16). Furthermore, we have used these 16S rRNA sequences to construct a phylogenetic tree based on other species of *Mycobacterium.* Furthermore, from the phylogenetic analysis of 16S rRNA sequences we show that these 8 uncharacterized CEI are new species of *Mycobacterium,* none of which are closely related to *M. ulcerans.*

2. MATERIALS AND METHODS

I. Eukaryotic Cell Culture:

L929 murine fibroblast cells (ATCC CCL1) were purchased from the American Type Culture Collection and passaged in Dulbecco modified Eagle medium supplemented with 5% heat-inactivated calf serum (Gibco BRL, Grand Island, New York)

II. Bacterial Culture:

All characterized species of *Mycobacterium* tested were obtained from the Trudeau Mycobacterial Culture Collection (TMCC) (National Institute of Health, Bethesda, Maryland) which had been stored at -80°C. Environmental mycobacteria were isolated from the Democratic Republic of Congo, Africa and were given as a gift from Francoise Portaels (Institute of Tropical Medicine, Antwerp, Belgium). These Congo environmental isolates (CEI) were received on Lowenstein-Jenson slants. Preliminary testing on these CEI had been done by Portaels (28). All mycobacteria tested were grown without shaking in Middlebrook 7H9 medium (Difco, Detroit, Michigan) supplemented with 10% Middlebrook oleic acid, bovine serum albumin, dextrose, and catalase enrichment (OADC supplement) at 37°C or 32°C (for CEI) in 175-cm^2 tissue culture flasks (Falcon).

III. Preparation of Acetone Soluble Lipids (ASL):

Extraction of ASLs from mycobacteria was performed as previously described (9). Briefly, 200 ml from late log phase cultures of all mycobacteria tested (TMCC and CEI) were centrifuged to pellet the bacteria. The supernatant was poured off, and the bacterial pellet was allowed to dry in an Isotemp Vacuum Oven (Model 280A; Fischer Scientific, Pittsburgh, Pennsylvania) at 65°C and 20 mHg. After drying, the bacterial pellet dry weight was determined using the Metler Toledo AT20 (Metler Toledo, Greifensee, Switzerland). The dried pellet was extracted with chloroform-methanol (2:1, vol/vol) for 2 h at room temperature. Cultures were once again spun to pellet the bacterial debris and separate the organic matter. The organic layer was removed and dried down with nitrogen (Pierce Reacti-Therm heating module; Rockford, Illinois) at 60°C and resuspended in ice-cold acetone.

IV. Separation and Analysis of ASLs by TLC:

Samples were analyzed by silica thin-layer chromatography (TLC) plates (Alltech, Deerfield, Illinois) using a solvent system of chloroform-methanol-water (90:10:1, vol/vol/vol) and lipids were visualized using ceric sulfate-ammonium molybdate in 2 M sulfuric acid stain as previously described (9). Briefly, 1 g of ceric sulfate hydrate (yellow salt) and 25 g of ammonium molybdate (colorless salt) are added to 450 ml of water. While stirring, 50 ml of concentrated sulfuric acid is added to the mixture and the mixture is stirred for 1 h at room temperature. The ASL weight was determined using the Metler Toldeo AT20 (Metler Toledo) scale. For the cytopathicity

assays, all samples were resuspended in 100% ethanol.

V. Cytopathicity Assays:

L929 cells were plated at 6 x 10^3 cells/well in 96-well tissue culture plates and allowed to adhere overnight at 37°C with CO_2. Assays were first performed with culture filtrate (CF) from the different mycobacteria, which was obtained by passing culture broth through a Steritop™ 0.22 μm pore filter (Milipore). This culture filtrate was used to detect the presence of any secreted molecules. Mycolactone is both a cell associated and secreted molecule, so its presence can be detected in both the CF and ASLs (9). Therefore, it was also necessary to extract the ASLs of the different mycobacteria. Extracted ASLs of mycobacteria were diluted with tissue culture medium to give a concentration of 20 μg/μl. Dilution of ASLs with tissue culture medium was done at a 1:2 ratio and serial dilutions were added to L929 cells at 1/20 of the volume. Ethanol (100%) as well as *M. ulcerans* 1615 ASLs, were used as the negative and positive controls, respectively. At 24 h and 48 h post treatment, cells were examined microscopically for cell destruction and detachment. Cytopathic activity (CPA) was determined as the minimum amount of ASL added (μg/ml) to cause 90% cell destruction and lifting of cells from the monolayer.

VI. ASL Separations of Cytopathic Mycobacteria:

Mycobacterial cultures determined to have a cytopathic effect were grown up in 1 liter of Middlebrook 7H9 using 850-cm^2 Roller Bottles (Corning). Strains were grown

without shaking at 32°C and 37°C for CEI and TMCC, respectively. ASLs were run in three different solvent systems: 90:10, 93:7, and 95:5 (chloroform-methanol, vol/vol) to determine which solvent system provided the best separation (Table 1). Preparative TLC was conducted using a 7924T Chromatotron Model (Harrison Research, Palo Alto, California) on a 2 mm silica glass backed plate (Alltech) (Figure 4). Briefly, solvent was added to a separatory funnel and allowed to run through the chromatotron until 25 ml of solvent was collected. Next, ASLs were resuspended in 2 ml of solvent, loaded into the chromatotron, and 15, 5 ml fractions of solvent elute were collected. The samples were analyzed by TLC and visualized with ceric sulfate-ammonium molybdate in 2 M sulfuric acid stain. Preparative TLC was used to further separate the lipid fractions. Briefly, ASLs were spotted across a TLC plate and run in a chloroform-methanol solvent system. Lipid fractions were scrapped from the plate and the silica was collected with 25-30 ml Sample Recovery Tubes (Alltech). Lipids were eluted out of silica using chloroform-methanol (90:10, vol/vol). Lipid fractions obtained by these methods were resuspended in 100% ethanol and tested on L929 murine fibroblasts for cytopathic activity.

VII. Mass Spectroscopy Analysis:

All MS analysis was performed by Richard Lee (University of Tennessee Health Science Center, Memphis, Tennessee)

Table 1. Solvent systems determined for chromatotron use for the *Mycobacterium* species with cytopathic lipids.

Species/ CEI Number	Solvent System Chosen (Chloroform-Methanol)
M. ulcerans	90:10
M. scrofulaceum	93:7
M. kansasii	95:5
01-627	95:5
01-628	93:7
01-629	93:7
01-632	95:5
01-633	93:7
01-636	95:5
01-664	95:5
01-665	93:7

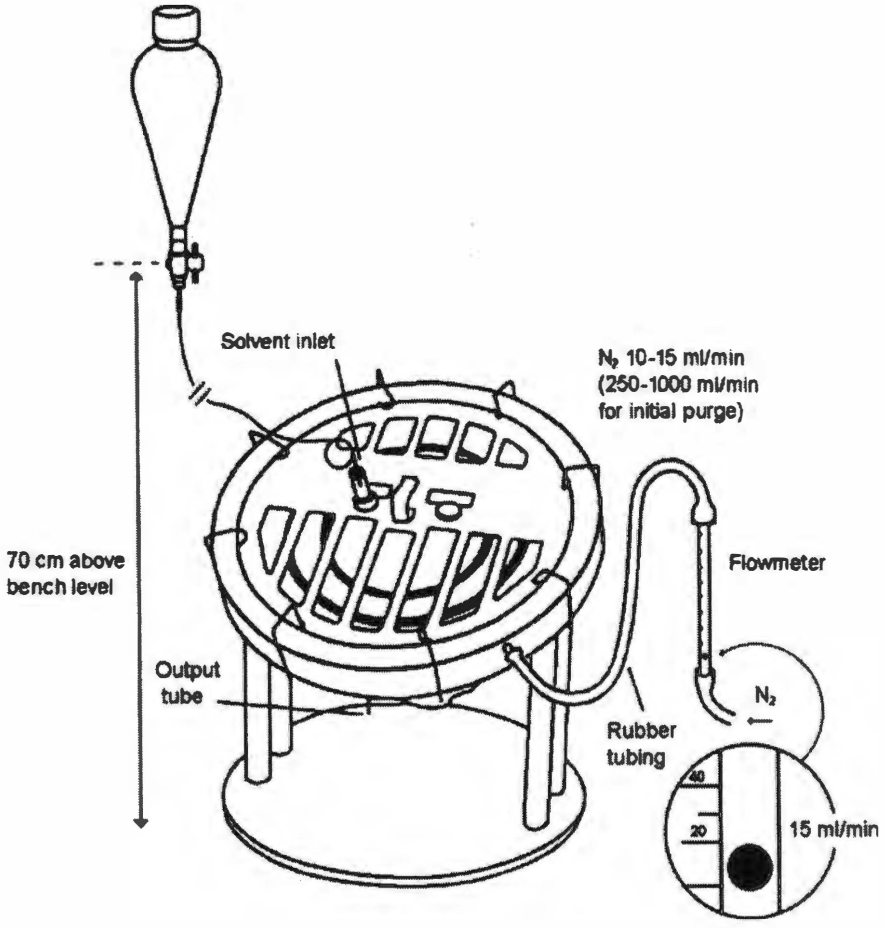


Figure 4. Chromatotron Model 7924T and funnel set-up diagram. Adapted from the instruction manual (Harrison Research, Palo Alto, California).

VIII. Genomic DNA Extraction:

Genomic DNA was extracted from the eight CEI with cytopathic ASLs (01-627, 01-628, 01-629, 01-632, 01-633, 01-636, 01-664, and 01-665) by using a previously described protocol (25). Briefly, 30 ml samples of the CEI were centrifuged at 3500 × g for 5 min at room temperature. The supernatant was removed and 5 ml of chloroform-methanol (2:1, vol/vol) was added to the wet pellet and vortexed for 2 min. Mixture was centrifuged again at 3500 × g and the solvents were poured off leaving the lipid lightened cells, and 1 ml of disruption solution (4 M guanidine thiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% (wt/vol) Sarkosyl, and 0.1 M 2-mercaptomethanol) was added and the mixture was vortexed for 1 min. The mixture was centrifuged at 4000 × g for 15 min and extracted with 1 vol phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). Sodium acetate (3 M) at 0.1 vol and isopropanol at 1 vol were added to the aqueous phase. Precipitation of DNA was performed by spinning the DNA at 1200 × g for 20 min at 4°C. Finally DNA was washed with 70% ethanol, dried, and resuspended in water. All reagents used were from Sigma (St. Louis, Missouri).

IX. Identification of Environmental Mycobacteria:

Environmental isolates were tested for the presence of IS2404 and IS2606 to determine whether they were *M. ulcerans*. PCR primers used for the IS elements (IS2404 and IS2606) in *M. ulcerans* were used as previously described with a slight modification to the PCR parameters (34). Briefly, the primers used to amplify the 492-bp region of IS2404 were MU5 (5'-AGC GAC CCC AGT GGA TTG GT-3') and MU6 (5'-CGG

TGA TCA AGC GTT CAC GA-3'). Primers used to amplify a 332-bp region of IS2606 were MU7 (5'-GGC CTG GCG GAT TGC TCA AGG-3') and MU8 (5'-CGT AGA TGT GGG CGA AAT GG-3') (34). Genomic DNA (1 µl) at a concentration of 50 ng/ml was added to 49 µl of the PCR amplification mixture to give a total of 50 µl, which contained 1 U of *Taq* DNA polymerase, (Promega Corporation, Madison, Wisconsin) 1 mM primers, 1.5 mM $MgCl_2$, and 1mM dNTPs (Promega). All PCR amplifications took place in a thermocycler (Eppendorf Scientific Inc., Westbury, New York). The reaction mixtures were subjected to 5 min of denaturation at 94°C and 30 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, followed by a final extension period of 72°C for 7 min and refrigeration.

In order to obtain taxonomic information from uncharacterized environmental mycobacteria, PCR of the 16S rRNA gene was performed. Genomic DNA was used for the PCR amplification of a region of the 16S rRNA gene, which is approximately 577-bp. These primers were designed from a region of the 16S rRNA gene from *M. tuberculosis* and were chosen because they are constructed from highly conserved regions within *M. tuberculosis* 16S rRNA gene. Also, within this 577-bp region are two hypervariable regions, A and B, which are used for species determination (17, 21). The primers used for this amplification, pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and MSHE (5'-GCG ACA AAC CAC CTA CGA G-3') had been previously described (16). For the PCR reactions 1 µl of genomic DNA was added to 49 µl of the PCR amplification mixture, with the final 50 µl volume containing 1 U of *Taq* DNA polymerase (Promega). The PCR amplifications were again performed in a thermocycler (Eppendorf) and the

amplifications were performed as 4 min of denaturation at 94°C and 40 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min, proceeded by a final extension period of 72°C for 7 min and refrigeration at 4°C.

X. PCR Product Analysis:

The PCR products (10 µl) were visualized using a 1% (wt/vol) agarose gel stained with ethidium bromide (5 µg/µl; Sigma). PCR products were then transformed using the TOPO TA Cloning® (Invitrogen Corporation, Carlsbad, California) kit using directions provided by the manufacturer. Clones containing the PCR product insert were screened using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-mediated blue-white discrimination and ampicillin resistance. White, ampicillin-resistant transformants were selected and grown overnight at 37°C with shaking in Luria-Bertani (LB) broth containing ampicillin (50 µg/ml). Minipreps were prepared from 5 ml cultures by using the Wizard® Plus Minipreps DNA Purification System (Promega). The minipreps were digested with EcoRI (New England Biolabs, Beverly, Massachusetts) and the digestion products were visualized on a 1% agarose gel (wt/vol) stained with ethidium bromide to determine which clones contained the insert. Clones containing the appropriate insert were grown in 25 ml volumes in LB broth plus ampicillin (50 µg/ml) overnight with shaking at 37°C. Wizard® Plus Minipreps DNA Purification System was used again to isolate plasmid DNA.

Bidirectional DNA sequences from the cloned fragments were obtained from the University of Tennessee Molecular Biology Sequencing Facility (Knoxville, Tennessee)

using the forward and reverse primers M13 (Invitrogen).

XI. Phylogenetic Analysis:

Alignments of 16S rRNA sequences were performed using ClustalW (Megalign, version 5; DNAStar, Madison, Wisconsin) and compared to the 16S rRNA sequences of 24 other *Mycobacterium* species as well as two *Nocardia* species. These 24 *Mycobacterium* species were chosen based on their homology to the CEI sequences determined from the BLAST search. Also, representatives of fast and slow growing *Mycobacterium* species were chosen based upon their range of diversity. *Nocardia* species were chosen as the outliers due to their close relationship to the *Mycobacterium* genera, without being a member of the genera. Alignments were analyzed using the program Molecular Evolutionary Genetics Analysis (MEGA) package version 2.1 (22). MEGA was also used to determine the bootstrap values, also, which were done with 500 permutations. A phylogenetic tree was constructed using the neighbor-joining (NJ) method with Jukes-Cantor distances, from the previously mentioned alignments. Partial 16S rDNA sequences from bp-1 to approximately bp-577, differing for all the CEI, were used for the alignments and phylogenetic analysis of the eight environmental isolates. *Nocardia asteroides* and *Nocardia farcinica* were used as outgroups to root the tree.

3. RESULTS

I. TLC Analysis of Mycobacteria:

Mycolactone is a light yellow uυ-active lipid with a ratio to front value (R_f) of 0.23 in chloroform-methanol-water (90:10:1, vol/vol/vol) and can be seen in lane 1 of the TLC analyses (11) (Figure 5, 6, and 7). TLC analysis of the Trudeau Mycobacterial Culture Collection (TMCC) species and of the Congo environmental isolates (CEI), showed that whereas many species contained components which were ultraviolet (uυ)-active there was no light yellow uυ-active component with a R_f similar to that of mycolactone run in chloroform-methanol-water (90:10:1, vol/vol/vol) (Data not shown). Since the core lactone lacks conjugated double bonds and is therefore not pigmented, it could not be detected by this method (24). TLC analysis showed that most of the mycobacteria tested contained many polar lipids. In fact, most of the mycobacteria tested contained a much greater quantity and variety of polar lipids than *M. ulcerans*. However, only *M. ulcerans* ASLs had a lipid species indicating the presence of mycolactone (Figure 5, 6, and 7).

II. Cytopathicity Assays:

ASLs were harvested from late log phase cultures (approximately 3 weeks for TMCC and 12 weeks for CEI) because this corresponded to the growth phase when macrolide production was at a peak in *M. ulcerans* (24). ASLs from 66% of the *Mycobacterium* species tested, or 19 out of 29 species, were not cytopathic

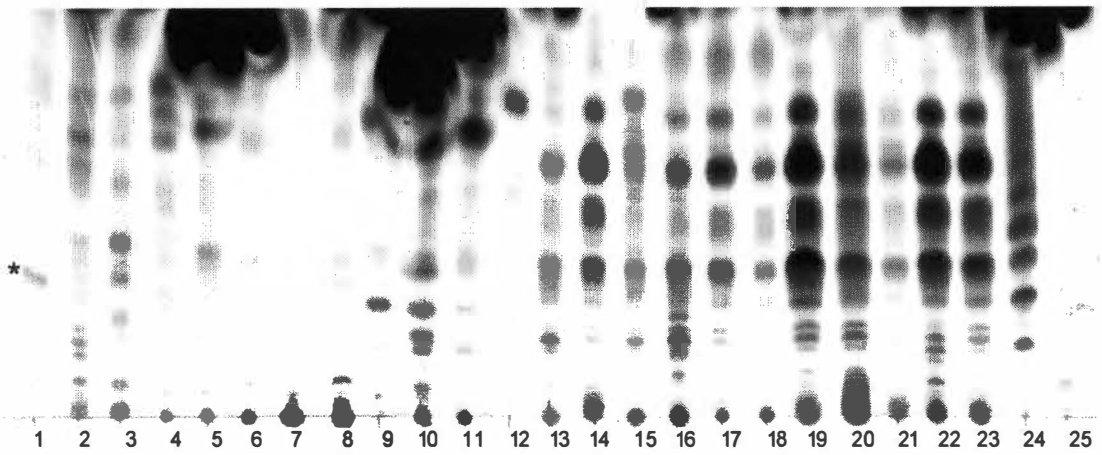


Figure 5. Silica TLC of ASLs extracted from the slow growing TMCC tested, run in chloroform-methanol-water (90:10:1, vol/vol/vol) and visualized by oxidative charring in a ceric molybdate—10% sulfuric acid stain. Strains are represented in lanes as follows: lane 1, 1615; lane 2, 706; lane 3, 1464; lane 4, 1467, lane 5, 1456; lane 6, 1318; lane 7, 1319; lane 8, 1324; lane 9, 1203; lane 10, 1204; lane 11, 1214; lane 12, 1601; lane 13, 1302; lane 14, 1305; lane 15, 1306; lane 16, 1307; lane 17, 1312; lane 18, 1314; lane 19, 1315; lane 20, 1316; lane 21, 1320; lane 22, 1321; lane 23, 1323; lane 24, 1481; lane 25, 1541. "*" indicates mycolactone A/B.

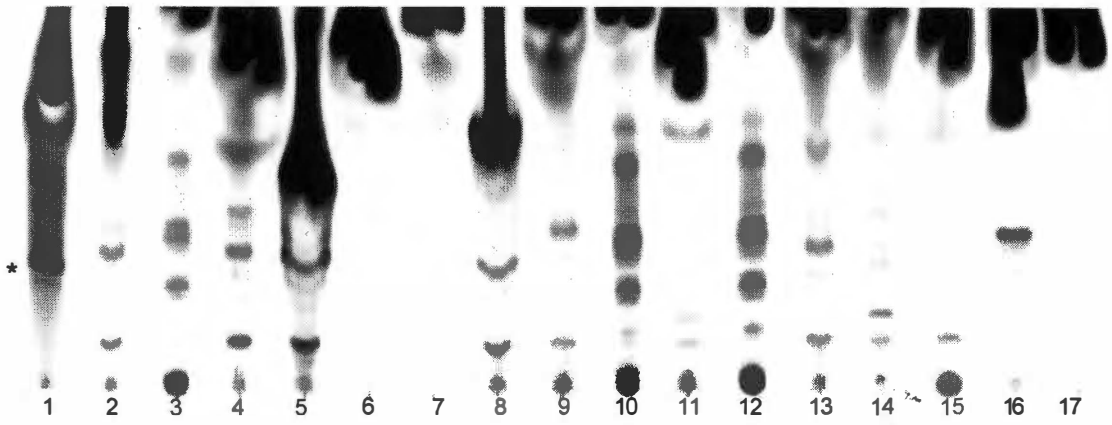


Figure 6. TLC of ASLs extracted from the slow growing CEI, run in chloroform-methanol-water (90:10:1, vol/vol/vol) and visualized by oxidative charring in a ceric molybdate—10% sulfuric acid stain. Strains are represented in lanes as follows: lane 1, 1615; lane 2, 01-626; lane 3, 01-627; lane 4, 01-628; lane 5, 01-629; lane 6, 01-630; lane 7, 01-631; lane 8, 01-632; lane 9, 01-633; lane 10, 01-634; lane 11, 01-635; lane 12, 01-636; lane 13, 01-664; lane 14, 01-665; lane 15, 01-666; lane 16, 01-667; lane 17, 01-668. "*" indicates mycolactone A/B.

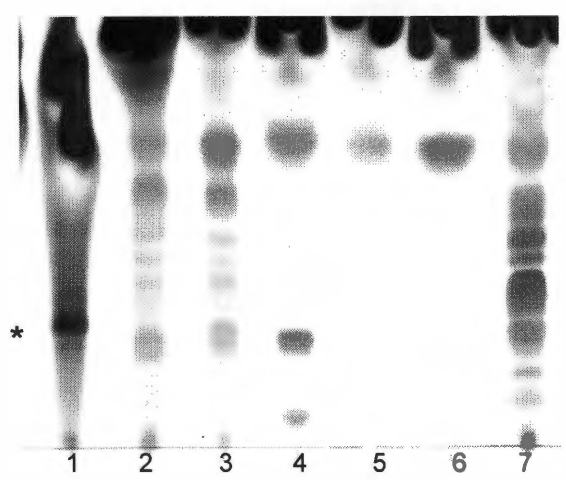


Figure 7. TLC of ASLs extracted from fast growing TMCC, run in chloroform-methanol-water (90:10:1, vol/vol/vol) and visualized by oxidative charring in a ceric molybdate—10% sulfuric acid stain. Strains are represented in lanes as follows: lane 1, 1615; lane 2, 1524; lane 3, 1543; lane 4, 1529; lane 5, 1530; lane 6 1516; lane 7, 1515. "*" indicates mycolactone A/B.

on L929 cells. None of the fast growing *Mycobacterium* species tested contained cytopathic lipids (Table 2). However, two slow growing, human pathogens, *M. scrofulaceum* and *M. kansasii*, did contain cytopathic ASLs. In addition it was surprising to find that, 8 out of the 16 CEI species tested (01-627, 01-628, 01-629, 01-632, 01-633, 01-636, 01-664, and 01-665) contained cytopathic ASLs (Table 3). However the cytopathic activity (CPA) of all of these lipids found differed greatly both in potency and cytopathic phenotype from that of mycolactone. Firstly, none of the lipids tested approached the potency of mycolactone. For example, cytotoxic ASLs from *Mycobacterium* species other than *M. ulcerans* have CPA values that are 10^5-10^7 higher than ASLs from 1615 *M. ulcerans*. Whereas 1615 *M. ulcerans* ASLs were cytopathic at 2.12 ng/ml, the cytopathic ASLs from other isolates exhibited a cytopathic activity at concentrations ranging from 2.00×10^4-3.30×10^2 μg/ml, values which require much more ASL to be added to see cytopathic activity. Therefore, 1615 *M. ulcerans* ASLs are more than 100,000 fold more active than any other cytopathic lipid (Table 3). Potency alone can not rule out the presence of mycolactone. Many more lipid fractions were isolated from some of the *Mycobacterium* species therefore the possibility exists that the molecule responsible for activity could be present in small amounts.

However the specific cytopathic effect produced by other mycobacterial ASLs differed both in terms of kinetics as well as in the morphological phenotype observed from that of mycolactone. Mycolactone mediated cytotoxicity developed slowly and cell rounding was not prominent for 18-24 hours. In contrast cytopathicity observed for ASLs from non-*M. ulcerans* bacteria developed much more rapidly and was obvious by

Table 2. Fast growing *Mycobacterium* species used in this study and the results of the ASL[a] cytopathicity assays for the fast growing mycobacteria.

TMC[b] Number and Species	Source	CPA[c] of ASL (µg/ml)
1615 *M. ulcerans* (ATCC 35840)	TMCC	0.0021
1524 *M. chelonae* (ATCC 35749)	TMCC	-[d]
1543 *M. chelonae* (ATCC 19977)	TMCC	-
1529 *M. fortuitum* (ATCC 6841)	TMCC	-
1530 *M. fortuitum* (ATCC 35754)	TMCC	-
1516 *M. phlei* (ATCC 35784)	TMCC	-
1515 *M. smegmatis* (ATCC 35797)	TMCC	-

[a] ASLs of a chloroform-methanol (2:1, vol/vol) extract
[b] TMC, Trudeau Mycobacterial Culture; TMCC, Trudeau Mycobacterial Culture Collection.
[c] CPA is defined as the minimal amount of ASLs added (µg/ml) to cause 90% cell destruction and detachment.
[d] "–", means no cytopathic effect seen at < 20 µg/µl.

Table 3. Slow growing *Mycobacterium* species used in this study and the results of the ASL[a] cytopathicity assays for the slow growing mycobacteria.

TMC[b] Number and Species Environmental Strain Number	Source	CPA[c] of ASLs (μg/ml)
1615 *M. ulcerans* (ATCC 35840)	TMCC	0.0021
706 *M. avium* (ATCC 35714)	TMCC	-[d]
1464 *M. intracellulare* (ATCC 35768)	TMCC	-
1467 *M. intracellulare* (ATCC 35770)	TMCC	-
1456 *M. gastri* (ATCC 15754)	TMCC	-
1318 *M. gordonae* (ATCC 35756)	TMCC	-
1319 *M. gordonae* (ATCC 35757)	TMCC	-
1324 *M. gordonae* (ATCC 14470)	TMCC	-
1203 *M. kansasii* (ATCC 35776)	TMCC	10000
1204 *M. kansasii* (ATCC 12478)	TMCC	1250
1214 *M. kansasii* (ATCC 35777)	TMCC	5000
1601 *M. microti* (ATCC 35781)	TMCC	-
1302 *M. scrofulaceum* (ATCC 35785)	TMCC	10000
1305 *M. scrofulaceum* (ATCC 35786)	TMCC	-
1306 *M. scrofulaceum* (ATCC 35787)	TMCC	10000
1307 *M. scrofulaceum* (ATCC 35788)	TMCC	10000
1312 *M. scrofulaceum* (ATCC 35790)	TMCC	20000
1314 *M. scrofulaceum* (ATCC 35791)	TMCC	10000
1315 *M. scrofulaceum* (ATCC 35792)	TMCC	20000
1316 *M. scrofulaceum* (ATCC 35793)	TMCC	20000
1320 *M. scrofulaceum* (ATCC 35794)	TMCC	-
1321 *M. scrofulaceum* (ATCC 35795)	TMCC	-
1323 *M. scrofulaceum* (ATCC 19981)	TMCC	-
1481 *M. nonchromogenicium* (ATCC 19530)	TMCC	-
1541 *M. flavescens* (ATCC 14474)	TMCC	-
01-626	CEI	-
01-627	CEI	2500
01-628	CEI	2500
01-629	CEI	10000
01-630	CEI	-
01-631	CEI	-
01-632	CEI	1600
01-633	CEI	10000
01-634	CEI	-
01-635	CEI	-
01-636	CEI	2500
01-664	CEI	2500
01-665	CEI	330
01-666	CEI	-
01-667	CEI	-
01-668	CEI	-

[a] ASLs of a chloroform-methanol (2:1, vol/vol) extract

[b] TMC, Trudeau Mycobacterial Culture; TMCC, Trudeau Mycobacterial Culture Collection; CEI, Congo Environmental Isolate obtained from Francoise Portaels.

[c] CPA is defined as the minimal amount of ASLs added (μg/ml) to cause 90% cell destruction and detachment.

[d] "–", means no cytopathic effect seen at < 20 μg/μl.

4-6 hours. Differences in cell morphology also clearly distinguished ASLs from *M. ulcerans* from other cytopathic lipids. Whereas mycolactone treated cells were characterized by eccentric nuclei and clear cytoplasm (Figure 9), vacuolization was the prominent feature produced by ASLs from non-*M. ulcerans* isolates (Figure 10 and Figure 11). Phenotypically, undisturbed L929 fibroblasts appeared as rhomboid cells lining the bottom of the well which formed a confluent monolayer by 48 h (Figure 8). Mycolactone had a readily identifiable and specific effect on L929 cells, characterized by cell rounding within 24 h, inhibition of cell growth, and detachment of the monolayer within 48 h (9) (Figure 9). None of the other lipids tested exhibited this phenotype. Most of the cytopathic ASLs shared a similar phenotype characterized by vacuole-like structures, which filled the cytoplasm of the cell. Two forms of vacuolization were detected. In one case extensive vacuolization occurred. This phenotype was seen with ASLs from *M. scrofulaceum* species, *M. kansasii* species, CEI 01-627, CEI 01-633, and CEI 01-636. These lipid samples produced many distinct vacuoles which filled the cytoplasm of the cell. Vacuoles could be detected as early as 6 h although maximum vacuolization did not occur until 24 h (Figure 10). A second phenotype, typified by CEI 01-628, CEI 01-629, CEI 01-632, CEI 01-664, and CEI 01-665, was characterized by simultaneous vacuolization and cell rounding by 4 h with the majority of cells being rounded and filled with vacuoles by 24 h. Eventually, cell lysis occurred and the vacuoles were seen free in the tissue culture media (Figure 11). Vacuolization does not occur in cells treated with *M. ulcerans* ASLs or purified mycolactone or mycolactone core (9, 10, 24). These results show that neither mycolactone nor the core mycolactone

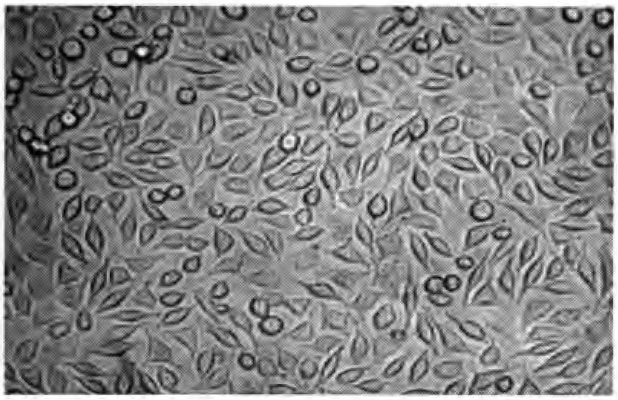


Figure 8. Undisturbed L929 fibroblasts. Picture was taken at 48 h and 200 × magnification.

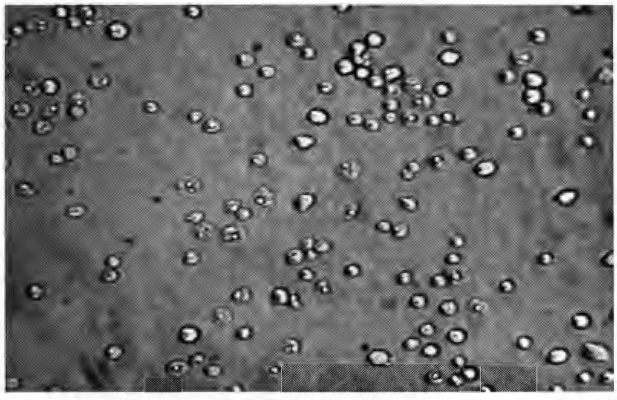


Figure 9. 1615 *M. ulcerans* ASLs added to L929 fibroblasts at a concentration of 2.12 ng/ml. Picture was taken at 48 h and 200 × magnification.

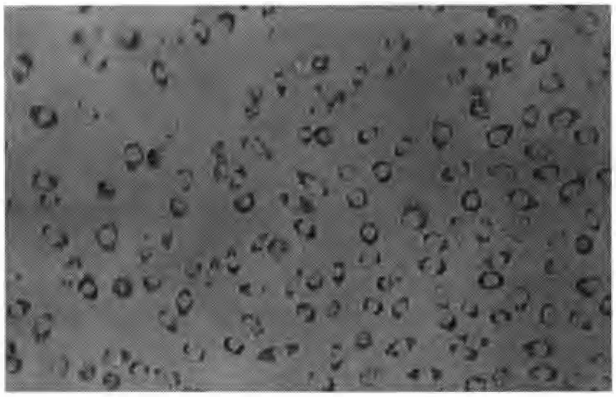


Figure 10. First typical phenotype of extensive vacuolization. Vacuolization seen here is with CEI 01-629 on L929 fibroblasts at 48 h and 200 × magnification.

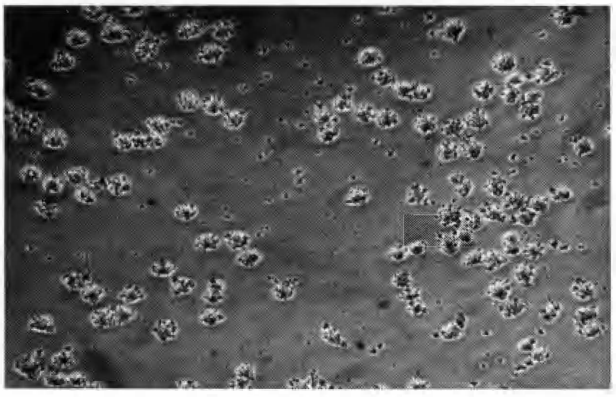


Figure 11. Second phenotype of simultaneous rounding and vacuolization. Vacuolization seen here with CEI 01-632 on L929 fibroblasts at 48 h and 200 × magnification.

lipid were present in any of the 46 *Mycobacterium* isolates tested.

III. Culture Filtrate Analysis:

George et al. have previously shown that mycolactone is secreted into the culture filtrate (CF) of the bacteria culture, and therefore, the CF of 1615 *M. ulcerans* exhibits a cytopathic effect on L929 cells with characteristic cell rounding and detachment (9). The CF of all other *Mycobacterium* species in this study were tested for CPA on L929 fibroblasts and none were cytopathic to L929 cells, even the species with slight cytopathic activity within their ASLs (Data not shown). However, ASLs from the CF of the non-*M. ulcerans* species of *Mycobacterium* were not extracted from the CF due to the lack of activity initially seen with their CFs. Most likely the cytopathic lipids are not secreted into the CF like mycolactone is, or these lipids are not potent enough to be detected. Also, the responsible lipid may be in the CF in quantities too small to be detected. In summary, the cytopathicity assays with ASLs and CF further suggest that out of the mycobacteria tested only *M. ulcerans* produces mycolactone.

IV. Purification of Cytopathic Lipids:

Cultures of *Mycobacterium* species which contained cytopathic ASLs were expanded to one liter to obtain sufficient lipids for analysis of individual lipid species. Unfortunately, we were unable to assign cytopathic activity to specific lipids in most cases due to the poor growth of many of the species and the small amounts of lipids produced. Many environmental mycobacteria grow very slowly and only at low

temperatures. In some cases 6 months of growth was required before cultures reached late log phase.

A specific cytopathic lipid was purified from CEI 01-628 ASLs by chromatotron separation (Figure 12). The cytopathic fraction was a lipid with a R_f value of 0.14 in a solvent system of chloroform-methanol (93:7, vol/vol) and a CPA of 2.5×10^3 μg/ml, which is the same CPA seen with whole ASLs of CEI 01-628. A lower CPA value than whole ASLs would be indicative of a pure lipid species, and this was not seen here. However, the cytopathicity phenotype on L929 cells of CEI 01-628 cytopathic lipid fraction matched the phenotype of whole ASLs (Data not shown).

The cytopathic fraction of CEI 01-628 was less polar than mycolactone, which has an R_f of 0.08 when run in chloroform-methanol (93:7, vol/vol). Also, CEI 01-628 cytopathic fraction lacked the potency of mycolactone (2.12 ng/ml), and specific phenotype seen with mycolactone as well as uv activity. Mass spectroscopy (MS) analysis was performed on this lipid component of CEI 01-628 to further distinguish it from mycolactone and the core lactone (Figure 13). Mycolactone produces a molecular ion at *m/z* 765.5 $[M+Na]^+$ whereas the core lactone is detected as a peak at *m/z* 447 $[M+Na]^+$ (10) (24) (Figure 14). Analysis of the cytopathic fraction of 01-628 shows that there is no peak at *m/z* 765.5 or at *m/z* 447 $[M+Na]^+$. Instead a major peak for CEI 01-628 was seen at *m/z* 279.1 $[M+Na]^+$ as well as several minor peaks. Therefore, it is quite obvious that this cytopathic fraction in CEI 01-628 is not mycolactone nor is the cytopathic fraction the core lactone. From the MS, TLC, and cell assay data we were able to confirm that the cytopathic lipid of CEI 01-628 was in fact not mycolactone or the

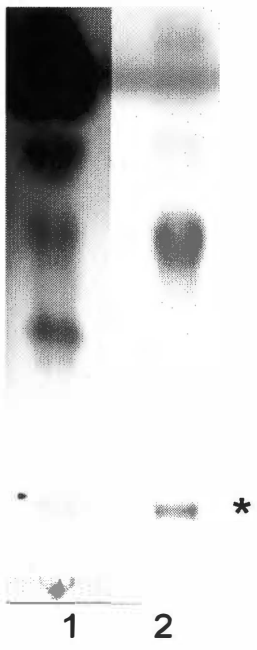


Figure 12. TLC analysis of CEI 01-628 ASLs from chromatotron spearation. Lane 1, 01-628 whole ASLs; lane 2, 01-628 ASL cytopathic fraction. "*", cytopathic lipid fraction. R_f value of cytopathic fraction is 0.14. TLC was run in chloroform-methanol (93:7, vol/vol) as the solvent system.

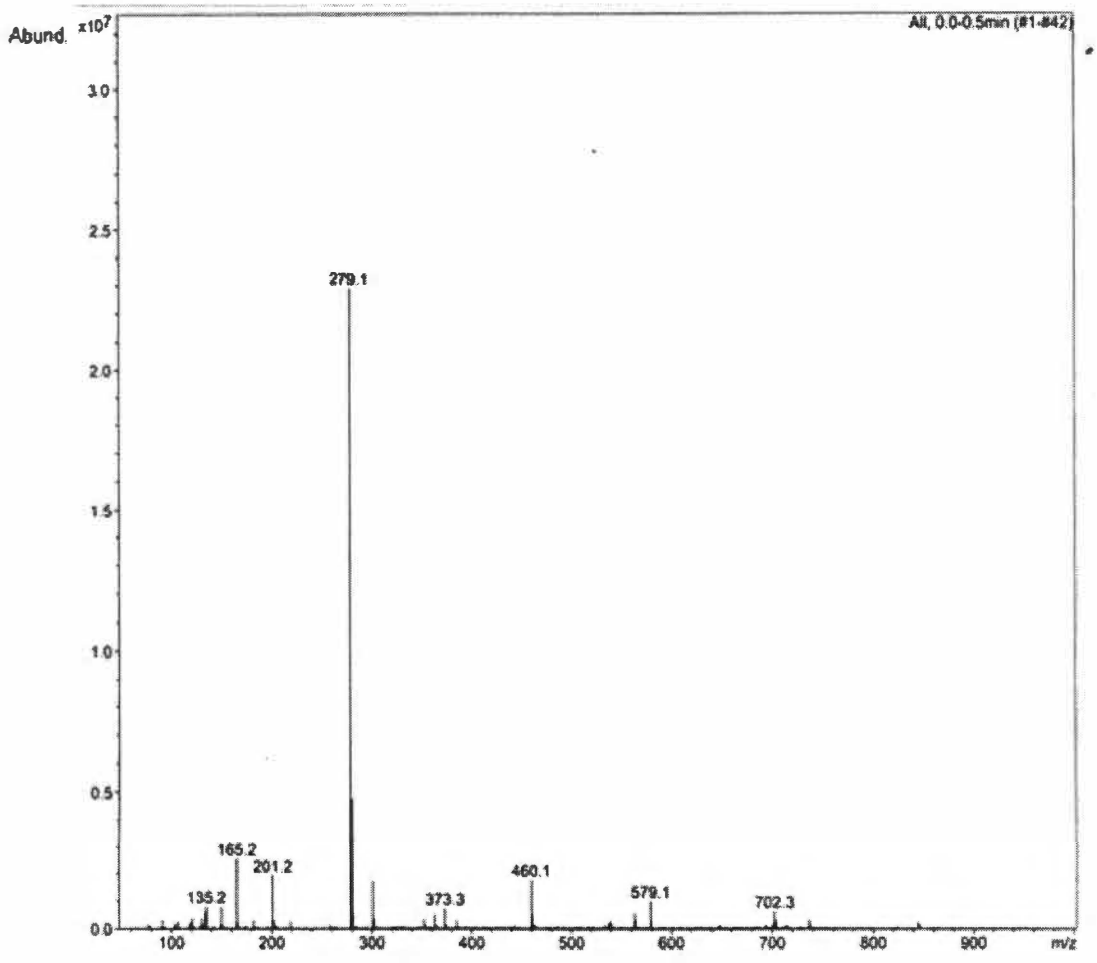


Figure 13. Mass Spectroscopy analysis of CEI 01-628 cytopathic fraction. The major species here occurs at a peak of *m/z* 279.1 $[M+Na]^+$.

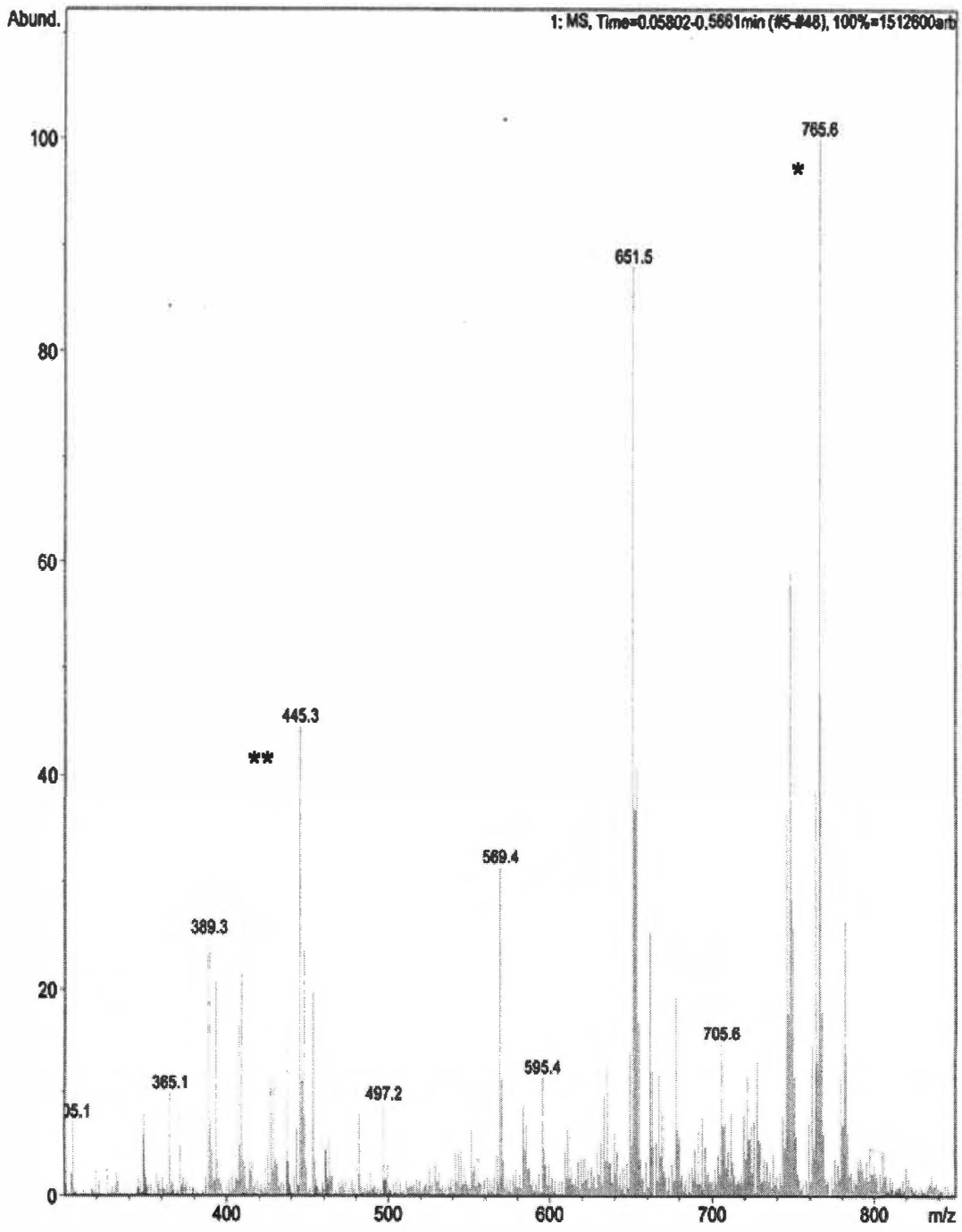


Figure 14. Mass Spectroscopy analysis of 1615 *M. ulcerans* ASLs. "*" indicates mycolactone, the most abundant species present. "**" indicates the core lactone.

core lactone. Furthermore, it can be concluded from the TLC data and the cell assay data that these other species of *Mycobacterium* that contain cytopathic lipids most likely do not contain mycolactone or the core lactone. More specifically, CEI 01-628 cytopathic fraction causes a vacuolization phenotype on L929 fibroblasts. This fraction was definitively identified as not mycolactone or the core lactone. Therefore, it can be concluded that because the other non-*M. ulcerans* species exhibit vacuolization too, they also do not contain mycolactone or the core lactone.

V. Identification of the 8 CEI with Cytopathic Lipids:

The CEI are slow growing, environmental mycobacteria isolated from water in a geographic area where *M. ulcerans* is endemic. All 16 CEI were isolated by Francoise Portaels (Institute of Tropical Medicine, Antwerp, Belguim) in the Democratic Republic of Congo, in the late 1970s. These strains were isolated from the Lower-Congo region, between Kinshasa and Moanda. These *Mycobacterium* isolates were determined to be slow growing mycobacteria with an optimal growth temperature of 33°C and sensitivity to dapsone. At the time of isolation, they were tested against all presently known species of *Mycobacterium* and did not match any known species in the late 1970s (28). Furthermore, tools for determining taxonomic relationships of new mycobacteria isolates have been more developed since then. In addition, the IS element based PCR diagnostic tool for *M. ulcerans* identification had not been developed when these species were isolated (34). Due to the fact the CEI were isolated in areas endemic to *M. ulcerans* infection, further characterization of these species was necessary. In addition it was

important to show that none of these CEI were *M. ulcerans*. The growth and colonial morphology of the CEI vary considerably; most isolates do not look phenotypically like *M. ulcerans* (Table 4). Also, these CEI were determined to have no growth at 37°C, but we have determined that poor growth occurs at 37°C (34) (Table 4).

In addition *M. ulcerans* contains two insertion sequences (IS2404 and IS2606) that are almost entirely unique to *M. ulcerans* (34). PCR detection of these two IS elements has become the gold standard used for diagnosis and identification of *M. ulcerans*. The possibility existed that some of these environmental isolates might represent *M. ulcerans* strains that do not have genes for mycolactone. Therefore, PCR was used to determine whether IS2404 or IS2606 were present in the 8 CEI with cytopathic lipids. Furthermore, because CEI species were isolated in aquatic habitats in areas endemic to *M. ulcerans*, we wanted to show that these species did not contain these IS elements found in *M. ulcerans* as this finding would strengthen the diagnostic validity of the PCR test on *M. ulcerans* (30). PCR results show that genomic DNA from *M. ulcerans* 1615 contains both the 492-bp fragment of IS2404 and the 332-bp fragment of IS2606. However, none of the CEI with cytopathic lipids contained either of these IS elements (Figure 15). Also, the remaining CEI (01-626, 01-630, 01-631, 01-634, 01-635, 01-666, 01-667, and 01-668) were tested for IS2404 and IS2606 by PCR. These species were also negative for both of these IS elements (Data not shown).

In order to further characterize the environmental isolates, partial sequencing of the 16S rRNA gene was done to determine the relationship of the uncharacterized CEI to other fast and slow growing species of *Mycobacterium*. First PCR was performed using

Table 4. Characterizations of uncharacterized slow growing mycobacteria from aquatic sources from the Democratic Republic of Congo.

Strain Number	Growth Morphology[a]/ Pigmentaion	Growth Temperatures		Cytopathicity[b]
		32°C	37°C	
1615 *M. ulcerans*	Smooth and Rough/Nonphotochromogenic[c]	+[d]	-	Yes
01-626	Rough/ Nonphotochromogenic	+	+/-	No
01-627	Smooth/ Scotochromogenic	+	+	Yes
01-628	Rough/ Nonphotochromogenic	+	+/-	Yes
01-629	Rough/ Photochromogenic	+	+/-	Yes
01-630	Smooth and Rough/ Scotochromogenic	+	+/-	No
01-631	Rough/ Scotochromogenic	+	+/-	No
01-632	Rough/ Nonphotochromogenic	+	+/-	Yes
01-633	Smooth and Rough/ Nonphotochromogenic	+	+/-	Yes
01-634	Smooth/ Scotochromogenic	+	+/-	No
01-635	Smooth/ Scotochromogenic	+	+/-	No
01-636	Smooth/ Scotochromogenic	+	+	Yes
01-664	Rough/ Nonphotochromogenic	+	+/-	Yes
01-665	Rough/ Scotochromagenic	+	+/-	Yes
01-666	Smooth/ Nonphotochromogenic	+	+/-	No
01-667	Smooth/ Scotochromogenic	+	+/-	No
01-668	Smooth and Rough/ Scotochromogenic	+	+/-	No

[a] Growth morphology as plated on Middelbrook 7H10 media.
[b] Cytopathicity on L929 fibroblasts.
[c] Nonphotochromogenic lacks the production of pigment with or without light present; Scotochromogenic produces pigment with or without light present; Photochromogenic produces pigment only after exposure to light. All pigment definitions taken from Kent et al. (20).
[d] "+" indicates growth; "-" indicates no growth; "+/-" indicates weak growth.

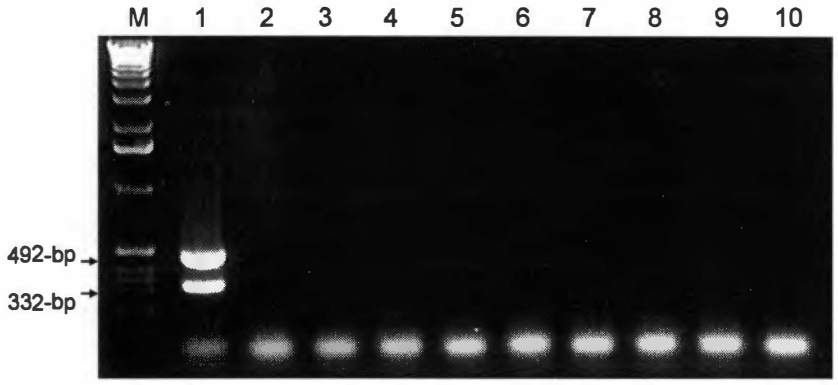


Figure 15. PCR analysis of 8 CEI with cytopathic lipids for IS2404 and IS2606. Lane M, 1-Kb ladder (Invitrogen); lane 1, *M. ulcerans*; lane 2, 01-627; lane 3, 01-628; lane 4, 01-629; lane 5, 01-632; lane 6, 01-633; lane 7, 01-636; lane 8, 01-664; lane 9, 01-665; lane 10, water. The 492-bp fragment represents IS2404 and the 332-bp fragment represents IS2606.

genomic DNA of these 8 CEI with cytopathic lipids, using primers based on bp 1-577 of the *M. tuberculosis* 16S rRNA gene (16S rDNA) as previously described (16). The primers used to amplify 16S rDNA in this study, pA and MSHE, were chosen because of the length of the fragment amplified and because they sequence two hypervariable regions in *Mycobacterium* species16S rRNA gene, A and B. Hypervariable region A is between bp 129-276 and hypervariable region B in between bp 430-495 using *Escherichia coli* 16S rDNA as a reference point for nucleotides. Both of these variable regions are useful in determining within species variations (21). All 8 of the CEI with cytopathic lipids as well as 1615 *M. ulcerans* were positive for the 16S rRNA fragment, which was amplified by primers designed from a 577-bp region of *M. tuberculosis*, whereas a water control was negative (Figure 16). The results of the PCR test further confirmed that these CEI were in fact species of *Mycobacterium*, and these PCR products were cloned and used for sequencing. The 16S rDNA obtained from sequencing was approximately 577-bp and contained both variable regions, A and B (Figure 17, 18, 19, 20, 21, 22, 23 and 24). The 8 different CEI 16S rDNA were all sequenced twice to determine accuracy. BLAST analysis showed that these sequences were unique mycobacterial sequences although they were closely related to 16S rRNA sequence for *Mycobacterium scrofulaceum*, *Mycobacterium celatum*, or *Mycobacterium cookii*. CEI 01-627 and CEI 01-636 16S rDNA sequences were 99 % (547 identical bases out of 552 for both CEI) homologous to *M. scrofulaceum* and CEI 01-629 was 96% (540 bases identical out of 558) homologous to *M. scrofulaceum*, according to the BLAST analysis. CEI 01-664 was 97% (534/549) homologous to *M. celatum*, while 01-632 and 01-628

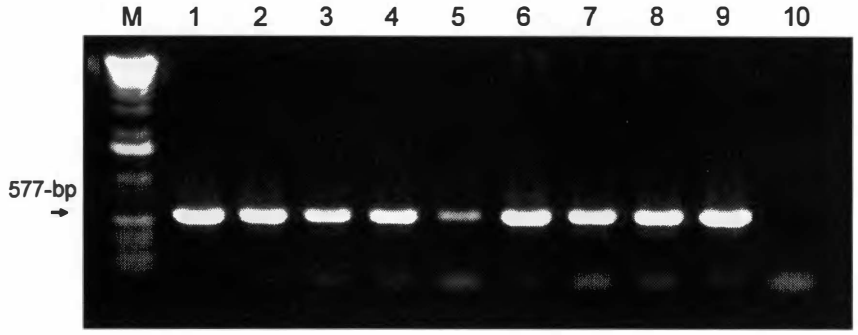


Figure 16. PCR analysis of 8 CEI with cytopathic lipids for the partial 16S rRNA fragment (577-bp). Lane M, 1-Kb ladder (Invitrogen); lane 1, *M. ulcerans*; lane 2, 01-627; lane 3, 01-628; lane 4, 01-629; lane 5, 01-632; lane 6, 01-633; lane 7, 01-636; lane 8, 01-664; lane 9, 01-665; lane 10, water.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GCC TCT TCG GAG GTA CTC GAG TGG **90**

CGA ACG GGT GAG TAA CAC GTG GGC AAT CTG CCC TGC ACT TCG GGA **135**

TAA GCC TGG GAA ACT GGG TCT AAT ACC GAA TAG GAC CAC TTG GCG **180**

CAT GCC TTG TGG TGG AAA GCT TTT GCG GTG TGG GAT GGG CCC GCG **225**

GCC TAT CAG CTT GTT GGT GGG GTG ATG GCC TAC CAA GGC GAC GAC **270**

GGG TAG CCG GCC TGA GAG GGT GTC CGG CCA CAC TGG GAC TGA GAT **315**

ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA **360**

ATG GGC GCA AGC CTG ATG CAG CGA CGC CGC GTG GGG GAT GAC GGC **405**

CTT CGG GTT GTA AAC CTC TTT CAC CAT CGA CGA AGG CTC ACT TTG **450**

TGA GTT GAC GGT AGG TGG AGA AGA AGC ACC GGC CAA CTA CGT GCC **495**

AGC AGC CGC GGT AAT ACG TAG GGT GCG AGC GTT GTC CGG AAT TAC **540**

TGG GCG TAA AGA GCT CGT AGG TGG TTT GTC GC **572**

Figure 17. Partial 16S rDNA sequence of CEI 01-627. Nucleotide numbers are located to the right of the sequence and are in bold.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GCC CTG CTT GCA GGG TGC TCG AGT **90**

GGC GAA CGG GTG AGT AAC ACG TGG GTG ATC TGC CCT GCA CTT CGG **135**

GAT AAG CTT GGG AAA CTG GGT CTA ATA CCG GAT AGG ACC ACA TGC **180**

TGC ATG GTG TGT GGT GGA AAG CTT TTT GCG GTG TGG GAT GGG CCC **225**

GCG GCC TAT CAG CTT GTT GGT GGG GTG ATG GCC TAC CAA GGC GAC **270**

GAC GGG TAG CCG GCC TGA GAG GGT GTC CGG CCA CAC TGG GAC TGA **315**

GAT ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC **360**

ACA ATG GGC GCA AGC CTG ATG CAG CGA CGC CGC GTG GGG GAT GAC **405**

GGC CTT CGG GTT GTA AAC CTC TTT CAC CAT CGA CGA AGG CCC GCG **450**

TTT GTG GGT TGA CGG TAG GTG GAG AAG AAG CAC CGG CCA ACT ACG **495**

TGC CAG CAG CCG CGG TAA TAC GTA GGG TGC GAG CGT TGT CCG GAA **540**

TTA CTG GGC GTA AAG AGC TCG TAG GTG GTT TGT CGC **576**

Figure 18. Partial 16S rDNA sequence of CEI 01-628. Nucleotide numbers are located to the right of the sequence and are in bold.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GCC CCT TTC GGG GGG TAC TCG AGT **90**

GGC GAA CGG GTG AGT AAC ACG TGG GTG ATC TGC CCT GCA CTT CGG **135**

GAT AAG CTT GGG AAA CTG GGT CTA ATA CCG GAT AGG ACT CTT GGC **180**

TGC ATG GTT GAG AGT GGA AAG CTT TTG CGG TGT GGG ATG GGC CCG **225**

CGG CCT ATC AGC TTG TTG GTG GGG TGA TGG CCT ACC AAG GCG ACG **270**

ACG GGT AGC CGG CCT GAG AGG GTG TCC GGC CAC ACT GGG ACT GAG **315**

ATA CGG CCC AGA CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA **360**

CAA TGG GCG CAA GCC TGA TGC AGC GAC GCC GCG TGG GGG ATG ACG **405**

GCC TTC GGG TTG TAA ACC TCT TTC ACC ATC GAC GAA GGC CCG CTT **450**

TTG GTG GGT TGA CGG TAG GTG GAG AAG AAG CAC CGG CCA ACT ACG **495**

TGC CAG CAG CCG CGG TAA TAC GTA GGG TGC GAG CGT TGT CCG GAA **540**

TTA CTG GGC GTA AAG AGC TCG TAG GTG GTT TGT CGC **576**

Figure 19. Partial 16S rDNA sequence of CEI 01-629. Nucleotide numbers are located to the right of the sequence and are in bold.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GCC CCT TTC GGG GGG TAC TCG AGT **90**

GGC GAA CGG GTG AGT AAC ACA TGG GTG ATC TGC CCT GCA CTT CGG **135**

GAT AAG CTT GGG AAA CTG GGT CTA ATA CCG GAT AGG ACC ACG GCC **180**

TGC ATG GGT TGT GGT GGA AAG CTT TTG CGG TGT GGG ATG GGC CCG **225**

CGG CCT ATC AGA TTG TTG GTG GGG TGA TGG CCT ACC AAG GCG ACG **270**

ACG GGT AGC CGG CCT GAG AGG GTG TCC GGC CAC ACT GGG ACT GAG **315**

ATA CGG CCC AGA CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA **360**

CAA TGG GCG CAA GCC TGA TGC AGC GAC GCC GCG TGG GGG ATG ACG **405**

GCC TTC GGG TTG TAA ACC TCT TTC ACC ATC GAC GAA GGC CCG CTT **450**

TTG GTG GGT TGA CGG TAG GTG GAG AAG AAG CAC CGG CCA ACT ACG **495**

TGC CAG CAG CCG CGG TAA TAC GTA GGG TGC GAG CGT TGT CCG GAA **540**

TTA CTG GGC GTA AAG AGC TCG TAG GTG GTT TGT CGC **576**

Figure 20. Partial 16S rDNA sequence of CEI 01-632. Nucleotide numbers are located to the right of the sequence and are in bold.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GCC CTT TCG GGG GTA CTC GAG TGG **90**

CGA ACG GGT GAG TAA CAC GTG GGT GAT CTG CCC TGC ACT TCG GGA **135**

TAA GCT TGG GAA ACT GGG TCT AAT ACC GGA TAG GAC TAC GAG TCG **180**

CAT GGC TTG TGG TGG AAA GCT TTT GCG GTG TGG GAT GGG CCC GCG **225**

GCC TAT CAG CTT GTT GGT GGG GTG ATG GCC TAC CAA GGC GAC GAC **270**

GGG TAG CCG GCC TGA GAG GGT GTC CGG CCA CAC TGG GAC TGA GAT **315**

ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA **360**

ATG GGC GCA AGC CTG ATG CAG CGA CGC CGC GTG GGG GAT GAC GGC **405**

CTT CGG GTT GTA AAC CTC TTT CAC CAT CGG CGA AGC CAG GGA CTT **450**

TTG TCT TCT GGT GAC GGT AGG TGG AGA AGA AGC ACC GGC CAA CTA **495**

CGT GCC AGC AGC CGC GGT AAT ACG TAG GGT GCG AGC GTT GTC CGG **540**

AAT TAC TGG GCG TAA AGA GCT CGT AGG TGG TTT GTC GC **578**

Figure 21. Partial 16S rDNA sequence of CEI 01-633. Nucleotide numbers are located to the right of the sequence and are in bold.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GCC TCT TCG GAG GTA CTC GAG TGG **90**

CGA ACG GGT GAG TAA CAC GTG GGC AAT CTG CCC TGC ACT TCG GGA **135**

TAA GCC TGG GAA ACT GGG TCT AAT ACC GAA TAG GAC CAC TTG GCG **180**

CAT GCC TTG TGG TGG AAA GCT TTT GCG GTG TGG GAT GGG CCC GCG **225**

GCC TAT CAG CTT GTT GGT GGG GTG ATG GCC TAC CAA GGC GAC GAC **270**

GGG TAG CCG GCC TGA GAG GGT GTC CGG CCA CAC TGG GAC TGA GAT **315**

ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA **360**

ATG GGC GCA AGC CTG ATG CAG CGA CGC CGC GTG GGG GAT GAC GGC **405**

CTT CGG GTT GTA AAC CTC TTT CAC CAT CGA CGA AGG CTC ACT TTG **450**

TGA GTT GAC GGT AGG TGG AGA AGA AGC ACC GGC CAA CTA CGT GCC **495**

AGC AGC CGC GGT AAT ACG TAG GGT GCG AGC GTT GTC CGG AAT TAC **540**

TGG GCG TAA AGA GCT CGT AGG TGG TTT GTC GC **572**

Figure 22. Partial 16S rDNA sequence of CEI 01-636. Nucleotide numbers are located to the right of the sequence and are in bold.

GGA CGA ACG CTG GCG GCG TGC TTA ACA CAT GCA AGT CGA ACG GAA **45**

AGG CCC CTT TCG GGG GGT ACT CGA GTG GCG AAC GGG TGA GTA ACA **90**

CGT GGG TGA TCT GCC CTG CAC TTC GGG ATA AGC TTG GGA AAC TGG **135**

GTC TAA TAC CGG ATA GGA CCA TGC CAT GCA TGT GGT GTG GTG GAA **180**

AGC TTT TGC GGT GTG GGA TGG GCC CGC GGC CTA TCA GCT TGT TGG **225**

TGG GGT GAT GGC CTA CCA AGG CGA CGA CGG GTA GCC GGC CTG AGA **270**

GGG TGT CCG GCC ACA CTG GGA CTG AGA TAC GGC CCA GAC TCC TAC **315**

GGG AGG CAG CAG TGG GGA ATA TTG CAC AAT GGG CGC AAG CCT GAT **360**

GCA GCG ACG CCG CGT GGG GGA TGA CGG CCT TCG GGT TGT AAA CCT **405**

CTT TCA CCA TCG ACG AAG GCC CGC TCT GCG GGT TGA CGG TAG GTG **450**

GAG AAG AAG CAC CGG CCA ACT ACG TGC CAG CAG CCG CGG TAA TAC **495**

GTA GGG TGC GAG CGT TGT CCG GAA TTA CTG GGC GTA AAG AGC TCG **540**

TAG GTG GTT TGT CGC **565**

Figure 23. Partial 16S rDNA sequence of CEI 01-664. Nucleotide numbers are located to the right of the sequence and are in bold.

AGA GTT TGA TCC TGG CTC AGG ACG AAC GCT GGC GGC GTG CTT AAC **45**

ACA TGC AAG TCG AAC GGA AAG GTC TCT TCG GAG ATA CTC GAG TGG **90**

CGA ACG GGT GAG TAA CAC GTG GGT GAT CTG CCC TGC ACT TCG GGA **135**

TAA GCT TGG GAA ACT GGG TCT AAT ACC GGA TAT GAT CAC ACG CTT **180**

CAT GGT GTG TGG TGG AAA GCT TTT GCG GTG TGG GAT GGG CCC GCG **225**

GCC TAT CAG CTT GTT GGT GGG GTA ATG GCC TAC CAA GGC GAC GAC **270**

GGG TAG CCG GCC TGA GAG GGT GTC CGG CCA CAC TGG GAC TGA GAT **315**

ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA **360**

ATG GGC GCA AGC CTG ATG CAG CGA CGC CGC GTG GGG GAT GAC GGC **405**

CTT CGG GTT GTA AAC CTC TTT CAC CAT CGG CGA AGC CGG ATG CTT **450**

TTG TGT CTG GTG ACG GTA GGT GGA GAA GAA GCA CCG GCC AAC TAC **495**

GTG CCA GCA GCC GCG GTA ATA CGT AGG GTG CGA GCG TTG TCC GGA **540**

ATT ACT GGG CGT AAA GAG CTC GTA GGT GGT TTG TCG C **577**

Figure 24. Partial 16S rDNA sequence of CEI 01-665. Nucleotide numbers are located to the right of the sequence and are in bold.

were 96% (550/572 and 551/573, respectively) homologous. Finally, 01-633 was 97% (546/558) homologous and 01-665 was 96% (538/557) homologous to *M. cookii.* All BLAST homologies had an expect value (E value) of 0.0, showing that these results were not due to chance. The results from the BLAST searches further indicated that these 8 CEI were not *M. ulcerans.* Furthermore, it confirmed that each of these 8 CEI were new species of *Mycobacterium.*

Alignments containing the partial sequence of the 8 CEI with cytopathic lipids were performed using 24 other *Mycobacterium* species (accession numbers are listed in Table 5) in order to place the new isolates in a taxonomic tree. These alignments were performed using ClustalW in the program Megalign (DNAStar). Variable regions within the 16S rRNA gene were important determinants of species differentiation (32). Hypervariable region A ranged from bp-129 through bp-276 and the CEI showed a significant amount of differentiation from known *Mycobacterium* species (Figure 25). Likewise, hypervariable region B (bp 430-495) also showed a significant amount of variation between the 8 CEI with cytopathic lipids as well as known *Mycobacterium* species (21) (Figure 26). These 16S rRNA hypervariable regions were very different for the CEI when compared to other *Mycobacterium* species and were a good indicator that the CEI were new species. A phylogenetic tree was constructed from these species with the MEGA program using the NJ method with Jukes-Cantor distances and *Nocardia asteroides* and *Nocardia farcinica* were used as outgroups to root the tree. Bootstrap values were calculated with 500 permutations, and those bootstrap values that were greater than 50, out of a total value of 100, were indicated with an asterisk (Figure 27).

Table 5. Accession numbers for *Mycobacterium* species used in the alignments for this study. Accession numbers for the different species of *Mycobacterium* were obtained from the GenBank Database.

Species	Accession Number
M. avium	AF306455
M. avium subsp. paratuberculosis	X52934
M. bovis	X55589
M. celatum	L08169
M. chelonae	X52921
M. cookii	AF480598
M. flavescens	X52932
M. gastri	X52919
M. gordonae	AJ536042
M. intracellulare	AJ536036
M. kansasii	AJ536035
M. leprae	X5399
M. marinum	AJ536032
M. nonchromogenicum	X52928
M. peregrinum	AF130308
M. phlei	AF480603
M. scrofulaceum	AJ536034
M. septicum	AF111809
M. simiae	X52931
M. smegmatis	AJ131761
M. terrae	X52925
M. tuberculosis	AJ131120
M. ulcerans	X58954
M. xenopi	AJ536033
N.. asteroides	X84850
N. farcinica	X91041

```
                129
M. tuberculosis TGATCTGCCCTGCACTTC-GGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGG-ACCACGGG-CTGCATG
M. ulcerans     C.................-.....................................-........-A......
M. scrofulaceum CA................-.....................................-.....TT.-GC.....
M. celatum      ..................-.........T.............................-....T...-A......
M. cookii       .................T-.....................................-..T....A-.......
01-665          ..................-.........T.........................T.-.T..CAC.-..T....
01-664          ..................-.........T.............................-....T.CC-A......
01-636          CA................-.................................A.....-.....TT.-GC.....
01-633          ..................-.........T.............................-..T...A.-TC.....
01-632          ..................-.........T.............................-.......C-.......
01-629          ..................-.........T.............................-..TCTT..-.......
01-628          ..................-.........T.............................-.....AT.-.......
01-627          CA................-.................................A.....-.....TT.-GC.....


                                                                                      276
M. tuberculosis TCTTG-TGGTGGAAAGCGCTTTAG----CGGTGTGGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGAC
M. ulcerans     ..C.-G...........--...T.----.............G.................................ACA
M. scrofulaceum C....-...........--...T.----.............G..................A..............T
M. celatum      .....-...........--...T.----.............G.................................T
M. cookii       GTC..-...........--...T.----.............G.................................T
01-665          GTG..-...........--...T----G...........G.................................T
01-664          .GG..-...........--...T----G...........G.................................T
01-636          C....-...........--...T----G...........G.................................T
01-633          G....-...........--...T----G...........G.................................T
01-632          GG...-...........--...T----G...........G..................A..............T
01-629          GT.GA-GA.........--...T----G...........G.................................T
01-628          GTG..-...........--...TT---G...........G.................................T
01-627          C....-...........--...T----G...........G.................................T
```

Figure 25. Alignment of the 16S rDNA for the 8 CEI with cytopathic lipids in the hypervariable region A (bp 129-277). Alignments were performed using the DNAstar program Megalign. CEI were compared with 24 other *Mycobacterium* species and 2 *Nocardia* species. The first nucleotide shown corresponds to *Escherichia coli* 16S rRNA position 129. Nucleotides different from *M. tuberculosis* are indicated. Dots represent identical nucleotides. Hyphens represent alignment gaps.

```
                 432                                                                  502
M. tuberculosis  CCTCTTTCACCATCGACGAAGGTCCGGGTTCTC---TCGGATTGACGGTAGGTGGAGAAGAAGCACCGGCC
M. ulcerans      ......................T....C.T..---.................................
M. scrofulaceum  .....................CT.A-----CTTT-GT..G.............................
M. celatum       .....................C.G.C....T..---CG.TGG...........................
M. cookii        ..............G.....CCGG.A.C.T.TGT-.TCTGG...........................
01-665           ..............G.....CCGGAT.C.T.TGTG.-CTGG...........................
01-664           .....................C.--C.C-TCTG---...G...........................
01-636           .....................CT--CAC--.TG---T.AG...........................
01-633           ..............G.....CCAG..AC.T.TGTC.TCTGG...........................
01-632           .....................C.--C.C.T.TGG--T..G...........................
01-629           .....................C.--C.C.T.TGG--T..G...........................
01-628           .....................C.--T.CGT.TG---T..G...........................
01-627           .....................CT--CAC.T.TG---T.AG...........................
```

Figure 26. Alignment of the 16S rDNA for the 8 CEI with cytopathic lipids in the hypervariable region B (bp 430-495). Alignments were performed using the DNAstar program Megalign. CEI were compared with 24 other *Mycobacterium* species and 2 *Nocardia* species. The first nucleotide shown corresponds to *Escherichia coli* 16S rRNA position 432. Nucleotides different from *M. tuberculosis* are indicated. Dots represent identical nucleotides. Hyphens represent alignment gaps.

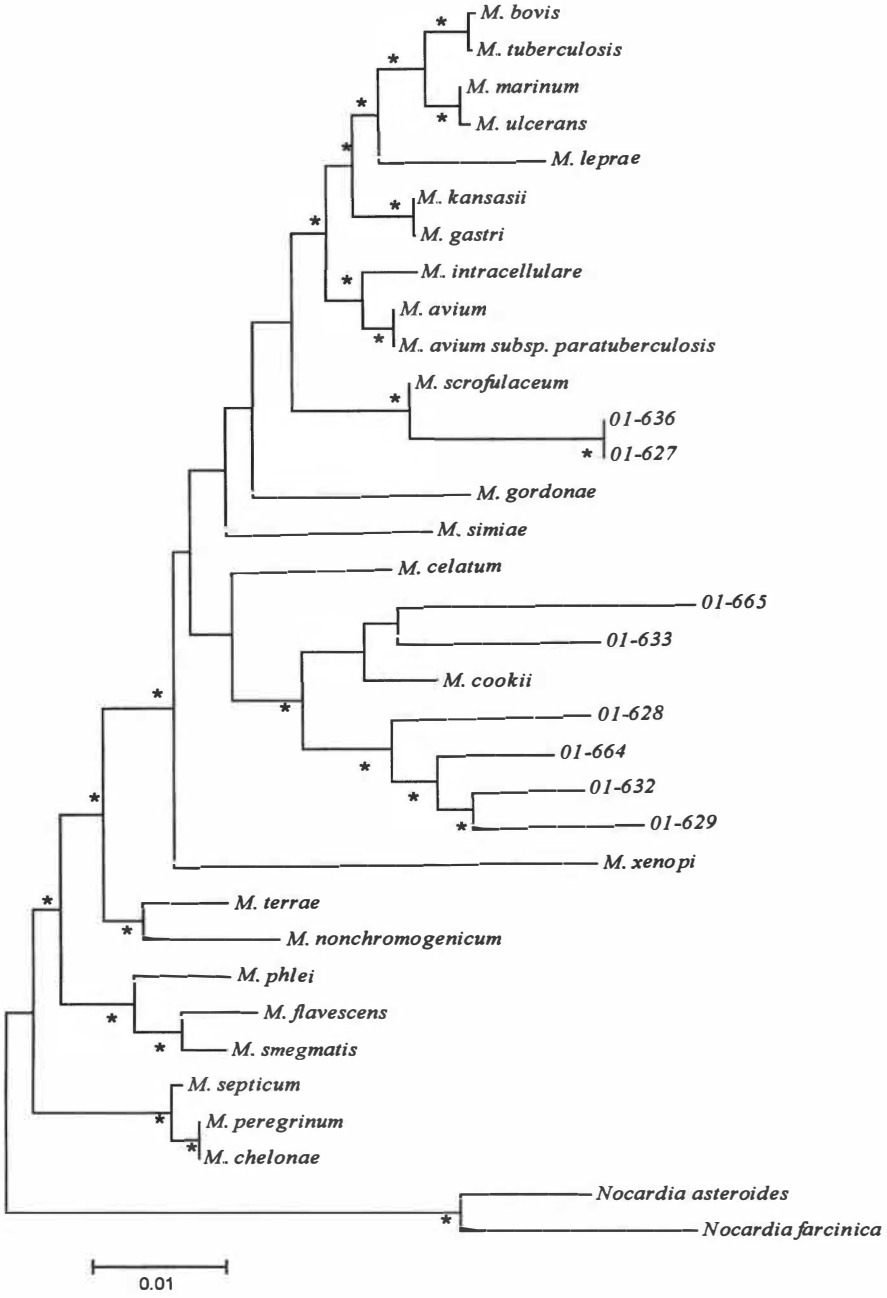


Figure 27. Phylogenetic tree constructed from 8 CEI with cytopathic ASLs. CEI 16S rDNA sequences were aligned with 24 other *Mycobacterium* species' 16S rDNA. *Nocardia* species were used as outgroups to root the tree. Bootstrap values were calculated with 500 permutations. "*" indicates a bootstrap value > 50.

Bootstrap values were an indication of the statistical relevance of a particular branch, or node, of the tree. It was quite apparent from the phylogenetic analysis that none of these 8 cytopathic CEI were closely related to *M. ulcerans*. However, three distinct groups of relationship were present. Two of the CEI, 01-636 and 01-627, were closely related to *M. scrofulaceum*. The other 6 CEI (01-628, 01-629, 01-632, 01-633, and 01-664) showed relationship to *M. cookii* and *M. celatum*, with 01-665 and 01-633 being more closely related to *M. cookii*.

The phylogenetic tree analysis of the CEI is consistent with the BLAST analysis, further proving the accuracy of the tree. So, it was determined that the tree is an accurate representation of the relationships of the 16S rRNA genes for different *Mycobacterium* species, particularly the uncharacterized CEI. Therefore, due to the BLAST analysis and the phylogenetic tree analysis, the conclusion was made that these 8 CEI with cytopathic lipids are new, novel species of *Mycobacterium* which are not closely related to *M. ulcerans*.

4. DISCUSSION

I. Importance of Study:

Previous work has identified mycolactone, a polyketide-derived macrolide, as a virulence determinant in *M. ulcerans* (10). Many macrolides are important biologically active molecules especially as antibiotics, antifungals, antiparasitics, immunosuppressants, etc. Until the discovery of mycolactone in *M. ulcerans*, macrolides had only been isolated from soil bacteria such as the *Streptomyces* and *Saccharopolyspora* species (19). *M. ulcerans* is an environmental organism, and the presence of numerous IS elements in this organism has led to the suggestion that mycolactone may have been acquired by horizontal transfer from another environmental organism (34). If these environmental organisms were unable to colonize a human host, it is unlikely that their lipids would have been tested for cytopathicity. However evidence presented in this study, shows that mycolactone is quite restricted to *M. ulcerans*, although other species of *Mycobacterium* do contain cytopathic lipids. Also, further findings from this study showed that a surprising number of uncharacterized mycobacteria from aquatic sources do produce cytotoxic lipids. Further examination of all *Mycobacterium* species would be necessary in order to say definitively that no other mycobacteria produce the macrolide, mycolactone.

Polyketides have such a wide range of biological activity and are very useful agents. Also, with the increase in genome mapping, many more PKS genes have been

identified in organisms such as *M. tuberculosis* (3). Therefore, the advent of new polyketides suggests new uses for such molecules especially in an age where microorganisms have increased resistance to almost all used antibiotics available. New drug discovery is becoming necessary in order to combat pathogens and polyketides have proven their worth biologically in the past (38). Therefore, a search for polyketides, such as mycolactone, in *Mycobacterium* species was an important study, although the results were negative.

II. Problems with Cytopathic Lipid Analysis:

Purification of mycolactone involved running *M. ulcerans* ASLs through a chromatotron or scrapping mycolactone off of a silica TLC plate. Therefore both chromatotron and preparative TLC methods were used to purify the cytopathic lipids. The solvent system determined to be best for mycolactone was chloroform-methanol (90:10, vol/vol). Chloroform-methanol-water (90:10:1, vol/vol/vol) is used for running *M. ulcerans* ASLs on TLC, however water is damaging to the chromatotron so it is left out during chromatotron use (9). However, several problems were encountered when using these separation methods. First, chromatotron separation was unsuccessful in separating these lipids due to the large numbers of lipid species and small quantity of individual lipids. Therefore the exact lipid species responsible for cytopathicity was not able to be isolated. Next preparative TLC was performed to try to achieve better separation. However problems arose again when trying to quantify these lipids. Most of these lipids were in such small amounts that they gave an undeterminable weight and the

L929 cell cytopathicity assays matched these results. All lipid species separated by preparative TLC had no cytopathic effect on L929 cells most likely due to the small amount of lipid present. Therefore, changes needed to be made to these separation methods. Mycolactone used a 1 mm glass silica plate (Alltech) for the separation of mycolactone. We originally used a 1 mm silica plate and it was unsuccessful. We then used a 2 mm silica glass plate (Alltech) in order to achieve better separation as well as using different combinations of chloroform-methanol. Separation with a 2 mm plate and using a different solvent system was more successful however it did not completely separate all lipid species. ASLs of the different *Mycobacterium* were able to be separated, but not into individual lipid species. Another problem that arose was more than one fraction would exhibit cytopathicity. So it was very likely that more than one lipid species was responsible for the cytopathic activity seen in the majority of the non-*M. ulcerans* species tested. Furthermore, when preparative TLC was used to further separate the chromatotron fractions the same quantitative problem arose.

Chromatotron separation was successful in isolating one particular cytopathic lipid species, CEI 01-628. The CPA of this particular lipid species matched the CPA of whole ASLs from CEI 01-628. Purity of a toxin is usually indicated by an increase in CPA when purified and this was not the case here. Several explanations can attribute to this occurrence. First, this lipid species may not be the only lipid responsible for the CPA seen. Secondly the lipid species may not be completely pure. However with either case we were able to determine that this lipid species is not mycolactone or core lactone. CEI 01-628 is one of the slow growing mycobacteria, and it usually takes three months for the

culture to reach late log phase growth. Furthermore the entire one liter culture is usually necessary to isolate the cytopathic lipid species; so working to isolate this lipid is difficult due to the growth time and quantity of lipid present. These constraints have also prohibited further identification tests to be performed on this lipid species.

Many pathogenic mycobacteria contain lipid determinants of virulence, such as *M. tuberculosis* and *M. leprae* (6, 31). Although the *Mycobacterium* species in this study lack the production of mycolactone or the core lactone, these cytopathic lipids could be molecules of biological significance. However, due to the small quantity of lipids present, we were unable to determine the lipid(s) responsible. A possible solution for isolating these particular lipids is to employ different methods of separation. In this study, solvents systems that were used contained different combinations of chloroform-methanol. However, different solvent systems using other solvents rather than just chloroform, methanol, and water should be tested. Solvent systems are difficult to work with and there are an abundance of solvents used for separating lipid species. Chloroform-methanol was used in this study due to its excellent separation of mycolactone. However, in this case, it may not be the best solvent for separation of the particular lipids in question. Mycolactone has a significant amount of cytopathic activity, therefore, the molecule was able to be isolated and assayed for cytopathicity. However, with the cytopathic lipids found here, most lipids were unable to be isolated, and assayed for cytopathicity due to lack of production of these lipids. These lipids lacked the potency that was seen with mycolactone therefore isolation was difficult, making any further analysis difficult as well. The lack of strong biological activity probably indicates

a lipid moiety that is only a secondary in virulence and is most likely not required for virulence.

III. Further Studies Concerning Environmental Isolates:

Included in this survey were 16 environmental mycobacteria isolated in the late 1970s by Francoise Portaels in the Democratic Republic of Congo in regions endemic to *M. ulcerans* (28). Although these 16 CEI were not identical to any of the *Mycobacterium* species at the time of isolation, many new diagnostic techniques have surfaced since then. Therefore, it was necessary for us to prove that these CEI were not species of *M. ulcerans* that lack the genes for mycolactone production. The PCR test showed that none of the 16 CEI had IS2404 or IS2606 which indicated that none of these species were *M. ulcerans*. Several of the CEI had cytopathic lipids and these species were further examined by 16S rRNA sequence analysis to determine their relationship to *M. ulcerans*. The 16S rRNA has been shown to be an effective method of determining species identity for mycobacteria. The 16S rRNA gene contains hypervariable regions amongst highly conserved regions. It is these hypervariable regions that are used to determine species (21). We used primers designed to amplify two hypervariable regions, A and B, to determine if these 8 CEI with cytopathic lipids are new *Mycobacterium* species (16). A phylogenetic tree was constructed with the 16S rDNA obtained, determining the relationships of the 8 CEI to other mycobacteria. However, several bootstrap values were below 50. These low bootstrap values could be due to the short sequence length. Attempts at amplifying the entire 16S rRNA gene were made and were

unsuccessful. Therefore, if more sequence data was obtained the bootstrap values might improve.

Only the 8 CEI with cytopathic lipids were phylogenetically analyzed. To further complete this study 16S rRNA sequence information should be obtained for the remaining CEI. Also, all of these CEI previously had biochemical tests performed on them, but in order to fully classify these species, further biochemical tests need to be performed (28).

IV. Conclusions:

From the results found in this study, several things were able to be concluded. First of all, none of the species tested in this study contain mycolactone or mycolactone congeners, based upon the cell assays. Furthermore, several species of *Mycobacterium* produce cytopathic polar lipids, but these lipids lack the potency seen with mycolactone and therefore were difficult to isolate. MS analysis on the one cytopathic lipid found (CEI 01-628) indicated that the cytopathic lipid is in fact not mycolactone or the core lactone. Furthermore, since CEI 01-628 cytopathic fraction produces vacuolization and most other species with cytopathic ASLs also exhibited vacuolization, it can be concluded that these cytopathic lipids are also not mycolactone or the core lactone. Finally, these uncharacterized environmental isolates of mycobacteria, isolated from aquatic environments endemic to *M. ulcerans* infection, are not *M. ulcerans.* However, they do represent new species of *Mycobacterium.* Some of these CEI are related to *Mycobacterium* species that are human pathogens, and their relevance as environmental

species would be interesting. However, further biochemical testing on these CEI is necessary to completely characterize these species.

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