JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1996, p. 962–965 Vol. 34, No. 4
0095-1137/96/$04.00+0

Variability in 3′ End of 16S rRNA Sequence of *Mycobacterium ulcerans* Is Related to Geographic Origin of Isolates

F. PORTAELS,[1]* P.-A. FONTEYNE,[1] H. DE BEENHOUWER,[1] P. DE RIJK,[1] A. GUÉDÉNON,[2] J. HAYMAN,[3] AND W. M. MEYERS[4]

Department of Microbiology, Division of Mycobacteriology, Institute of Tropical Medicine, B-2000 Antwerp, Belgium[1]; Lazaret-Akpakpa, Cotonou, Benin[2]; Box Hill Hospital, Victoria, Australia[3]; and Armed Forces Institute of Pathology, Washington, DC 20306[4]

Received 7 July 1995/Returned for modification 25 September 1995/Accepted 3 January 1996

Mycobacterium ulcerans **causes extensive ulcers (Buruli ulcers) in the skin of humans. Analysis of the 3′-terminal region of the 16S rRNA gene sequence of 17 strains of *M. ulcerans* from Africa, the Americas, and Australia revealed three subgroups corresponding to the continent of origin, and some variable phenotypic characteristics. This sequence is useful for the rapid detection of *M. ulcerans* and discriminates *M. marinum* and *M. shinshuense* from *M. ulcerans*.**

Mycobacterium ulcerans, a slowly growing mycobacterium with optimal growth at 33°C, causes chronic necrotizing ulcers in the skin of humans (15, 19, 20) and other mammals (16).

M. ulcerans infection, or Buruli ulcer (BU), was first described in Bairnsdale, Australia, in 1948 (13) and was subsequently reported in numerous, mostly tropical, countries in Africa, the Americas, Southeast Asia, and the central Pacific (3). Recent reports mention increases in the incidence of BU in Benin (12), Australia (6, 11), and Côte d'Ivoire (14). These observations have revived interest in the microbiology of *M. ulcerans* and the epidemiology, early diagnosis, and treatment of BU. The epidemiology of BU is poorly understood, but most foci are associated with slowly flowing or stagnant waters; however, *M. ulcerans* has never been recovered from the environment (18, 20).

BU is rarely detected in an early and readily curable stage. Most lesions are widely ulcerated when they are detected and require extensive surgical excision and skin grafting. Chemotherapy of advanced ulcers is disappointing. Patients are frequently improperly managed and suffer permanent disability, with devastating personal consequences. Diagnosis of preulcerative or early ulcerative stages greatly improves the efficacy of treatment. Primary culture of *M. ulcerans* under optimal conditions may take several months (23). Rapid molecular identification of the causative organism in tissues or exudates would allow appropriate early therapy.

Information on the molecular characterization of *M. ulcerans* is scarce. Jackson et al. (10) identified and differentiated *M. ulcerans* strains on the basis of restriction fragment length polymorphisms using the pTBN12 probe. Sequences of 16S rRNA are included in databases (EMBL accession numbers X58954 and Z13990). The two sequences are very similar to the sequence published for *M. marinum* (22). A single-base-pair variation at position 1248 distinguishes *M. ulcerans* from *M. marinum* (Table 1) and has been proposed as an identifying criterion for separating these two species (8). Other significant differences are observed between nucleotide sequences of *M. ulcerans* X58954 (ACCCTTTTTTGGG), *M. ulcerans* Z13990 (ACCCTTTGGG), and *M. marinum* (ACCTTTGGG) at positions 1450 to 1452 (Table 1).

A new mycobacterium, *M. shinshuense* (26), isolated only from the skin ulcer of a patient in Japan (24), is phenotypically related to *M. ulcerans* (25), but this close relationship was never studied by sequence analysis.

These findings are interesting in that they suggest (i) genetic heterogeneity within the species *M. ulcerans* and (ii) close phylogenetic relationships between *M. ulcerans*, *M. marinum*, and *M. shinshuense*.

The present study was undertaken to determine (i) if the genetic heterogeneity of *M. ulcerans* is related to other characteristics of the species and to the geographic origins of the isolates, (ii) if this heterogeneity may identify different subgroups within the species, and (iii) if the 3′ end sequences of the 16S rRNA genes differentiate the three species (*M. ulcerans*, *M. marinum*, and *M. shinshuense*) and could later serve as a clinically useful means of identifying the three species, even in early stages of disease.

Twenty-one strains were studied (Table 2), including reference strains of *M. ulcerans* (ATCC 19423), *M. marinum* (ATCC 927), and *M. shinshuense* (ATCC 33728). Twelve *M. ulcerans* strains were isolated at the Institute of Tropical Medicine (ITM) from biopsy specimens from patients (10 African and 2 Australian patients). Two strains were contributed by D. Dawson (Laboratory of Microbiology and Pathology, Queensland Health, Brisbane, Australia), and two isolates from the same patient came from Mexico. The two other strains were *M. marinum* 8231, isolated in Belgium from a cutaneous granuloma, and *Mycobacterium* sp. strain 842, which is phenotypically related to *M. ulcerans* and which was obtained from a granulomatous lesion of a patient from Suriname living in Holland (27).

Phenotypic identification tests were performed as described by Vincent Lévy-Frébault and Portaels (28).

By using bacterial lysates, a 517-bp fragment of the 16S rRNA gene was amplified and sequenced by a previously described method (21).

The primers used for amplification and sequencing were rRog (biotinylated or not) (positions 1542 to 1521; 5′-AAGG AGGTGATCCAGCCGCA-3′) and 1004R (positions 985 to 1004; 5′-AGGAATTCTGGGTTTGACATGCACAGGA-3′). These same primers and the following three primers were used for sequencing: G1140 (positions 1119 to 1140; 5′-TCATGT

* Corresponding author. Mailing address: Department of Microbiology, Division of Mycobacteriology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium.

TABLE 1. Variable regions in the 3′ end sequences of 16S rRNAs of *M. ulcerans* and related species

Organism	Residue at the following positions[a]:		
	1248	1289	1450–1452
M. ulcerans[b]	CGGTGCAAAG	TTTAAAGCCG	ACCC---TTTGGG
M. marinum[c]	····A·····	·····A····	··········
M. marinum, ITM type	····A·····	·····A····	···C------
M. ulcerans, ITM type 1	····G·····	·····C····	···CTTT····
M. ulcerans, ITM type 2	····G·····	·····C····	···C------
M. ulcerans, ITM type 3	····G·····	·····A····	···C------
M. shinshuense	····G·····	·····G····	···C------

[a] The positions are given for the noncoding strand according to the sequence of the *E. coli* 16S rRNA gene (1).
[b] The *M. ulcerans* sequence has been introduced by Hofer et al. (8) into the EMBL database under accession number Z13990.
[c] The *M. marinum* sequence has been published by Rogall et al. (22).

TGCCAG-CACGTAATGGT-3′), S1366 (positions 1348 to 1366; 5′-ATCGCAGATCAGCAACGC-3′), and R1333 (positions 1350 to 1333; 5′-GATTACTAGCGACTCCGA-3′).

The nucleotide positions indicate the target sites of the primers in 16S rRNA as represented in the sequence of *Escherichia coli* (2). Primer rRog has been described by Böddinghaus et al. (1). The underlining indicates the *Eco*RI restriction site.

The phenotypic characteristics of *M. ulcerans*, *M. marinum*, *M. shinshuense*, and the *Mycobacterium* sp. isolate are given in Table 3. Characteristics of African, Australian, and American strains of *M. ulcerans* are presented separately and are compared with previously published data (28). Some phenotypic characteristics seem to differentiate African, Australian, and American strains; six African (60%), two North American (100%), and only one Australian strain (20%) were positive for growth on *p*-nitrobenzoate, and acid phosphatase activity was positive for seven African strains (70%) but was negative for all other strains. Given the few *M. ulcerans* strains investigated from each geographic area, the phenotypic differences between strains of different origins should be confirmed with more strains from other foci and with a larger variety of phenotypic tests. *M. shinshuense* also differed from *M. ulcerans* and *M. marinum*. These findings agree with previously published data (24, 25). Unclassified strain *Mycobacterium* sp. strain 842 shared several properties with *M. shinshuense* (Table 3) but differed from this species, as did *M. ulcerans*, by high catalase activity. *Mycobacterium* sp. strain 842 resembled Australian isolates of *M. ulcerans* but differed by pigmentation in the dark.

Comparisons of the sequences obtained in the present study with those published previously are given in Table 1. The polymorphism between the two *M. ulcerans* sequences previously included in the EMBL database was confirmed by the sequences obtained in the present study for strains of different origins. Indeed, at positions 1450 to 1452, the sequences of the 10 African strains (ITM type 1) were identical to the sequence of strain X58954 and the sequences of 5 Australian strains (ITM type 2) were identical to the sequence of strain Z13990. The two Mexican strains showed another type (ITM type 3), characterized by an A at position 1289, which was also seen in *M. marinum*, whereas the Australian and African *M. ulcerans* strains presented a C at this position.

The 16S rRNA sequence of *M. marinum* revealed identical sequences for the two tested strains, but at positions 1450 to 1452 the sequence differed from the sequence of *M. marinum* published by Rogall et al. (22). *Mycobacterium* sp. strain 842

TABLE 2. Origin and sequencing results of *M. ulcerans*, *M. marinum*, *M. shinshuense*, and one unclassified *Mycobacterium* sp.

ITM no.	Species identification	Origin	Source	Sequencing results
842	*Mycobacterium* sp.	Suriname	P. H. J. Van Keulen, strain 701357	*M. marinum*, ITM type
1837	*M. ulcerans*	Ghana	ITM	*M. ulcerans*, ITM type 1
3129	*M. ulcerans*	Zaire	ITM	*M. ulcerans*, ITM type 1
5114	*M. ulcerans*	Mexico	P. Lavalle	*M. ulcerans*, ITM type 3
5143	*M. ulcerans*	Mexico	P. Lavalle	*M. ulcerans*, ITM type 3
5147	*M. ulcerans*	Australia	ATCC 19423	*M. ulcerans*, ITM type 2
5151	*M. ulcerans*	Zaire	ITM	*M. ulcerans*, ITM type 1
5152	*M. ulcerans*	Zaire	ITM	*M. ulcerans*, ITM type 1
5153	*M. ulcerans*	Zaire	ITM	*M. ulcerans*, ITM type 1
5155	*M. ulcerans*	Zaire	ITM	*M. ulcerans*, ITM type 1
7732	*M. marinum*		ATCC 927	*M. marinum*, ITM type
8231	*M. marinum*	Belgium	ITM	*M. marinum*, ITM type
8756	*M. shinshuense*	Japan	ATCC 33728	*M. shinshuense*
8849	*M. ulcerans*	Australia	ITM	*M. ulcerans*, ITM type 2
9146	*M. ulcerans*	Benin	ITM	*M. ulcerans*, ITM type 1
9540	*M. ulcerans*	Australia	D. Dawson, strain 11098	*M. ulcerans*, ITM type 2
9550	*M. ulcerans*	Australia	D. Dawson, strain 17679	*M. ulcerans*, ITM type 2
94-339	*M. ulcerans*	Australia	ITM	*M. ulcerans*, ITM type 2
94-511	*M. ulcerans*	Benin	ITM	*M. ulcerans*, ITM type 1
94-512	*M. ulcerans*	Benin	ITM	*M. ulcerans*, ITM type 1
94-886	*M. ulcerans*	Benin	ITM	*M. ulcerans*, ITM type 1

TABLE 3. Sequencing results and phenotypic characteristics of *M. marinum*, *M. ulcerans*, *M. shinshuense*, and one unclassified *Mycobacterium* sp.

Parameter	*M. marinum*[a]	*M. ulcerans*[a]	No. of strains				
			M. ulcerans positive[b]			*M. shinshuense*	*Mycobacterium* sp. strain 842
			AF	AU	AM		
No. of strains tested			10	5	2	1	1
Pigmentation in the dark	−	−	0	0	0	1	1
Pigmentation in the light	+	−	0	0	0	1	1
Growth at 37°C	−	−	0	0	0	0	0
Growth on peptone agar	+	−	0	0	0	0	0
Growth in presence of:							
Isoniazid (10 μg/ml)	M	M	8	4	2	0	0
Thiophene-2-carboxylic hydrazide	+	+	10	5	2	0	1
Hydroxylamine (250 μg/ml)	+	F	7	5	2	0	1
p-Nitrobenzoate (500 μg/ml)	M	−	6	1	2	0	0
NaCl 5%	−	−	0	0	0	0	0
Enzymatic properties							
Catalase, >45 mm of foam	F	−	0	0	0	1	0
Tween 80 hydrolysis (10 days)	+	−	0	0	0	0	0
Urease activity	+	F	3	0	0	0	0
Niacin production	−	−	4	1	0	0	0
Nitrate reduction	−	−	0	0	0	0	0
Acid phosphatase activity	+	−	7	0	0	0	0
Mycolate types							
α-Mycolates	+	+	10	5	2	1	1
α′-Mycolates	−	−	0	0	0	0	0
Methoxymycolates	+	+	10	5	2	1	1
Ketomycolates	+	+	10	5	2	1	1
ω-Carboxymycolates	−	−	0	0	0	0	0
Sequencing results			Type 1	Type 2	Type 3	*M. shinshuense*	*M. marinum*

[a] Data are from Vincent Lévy-Frébault and Portaels (28); +, >85% of the strains were positive; −, <15% of the strains were positive; M, 50 to 85% of strains positive; F, 15 to 49% of strains positive.
[b] Strains isolated from African (AF), Australian (AU), and American (AM) patients.

showed a sequence identical to those of our two *M. marinum* strains. *M. shinshuense*, like *M. ulcerans*, had a G at position 1248, but it differed from our three *M. ulcerans* types by a G at position 1289. For positions 1450 to 1452, identical results were obtained for *M. shinshuense*, our *M. marinum* strain (ITM type), and the *M. ulcerans* strains from Australia and America. If a reading error is excluded in the sequence of *M. marinum* published by Rogall et al. (22), *M. marinum*, *M. ulcerans*, and *M. shinshuense* showed at least six different sequences for the 3′ end of the 16S rRNA. All *M. ulcerans* 16S rRNA sequences differed from those of *M. marinum* at position 1248.

We observed an invariable correlation between the geographic origins of the strains and polymorphisms in the 16S rRNA gene. These results agree with the previously proposed classification based on restriction fragment length polymorphism analysis (10).

The significance of the variabilities in 16S rRNA sequences in the phylogeny and genotyping of *M. ulcerans* is not clear. Partial sequencing of 16S rRNA is insufficient to establish a valid phylogenetic tree. The sequence at positions 1450 to 1452 shows a similarly important polymorphism in the *M. avium* complex. In the *M. avium* complex, polymorphism is more extensive in other regions of the 16S rRNA sequence (1).

The associations between phenotypic and genotypic variations in *M. ulcerans* isolates and virulence or pathogenicity need investigation. Why, for example, calcification is more prominent in lesions in Africans than in Australians (7) is unknown. There is wide acceptance that most histopathologic changes in BU lesions are caused by a cytotoxic agent and, possibly, an immunosuppressive agent elaborated by *M. ulcerans* (15, 17). We have noted wide variations in the production of these agents by different isolates but have not compared strains from different geographic areas with known phenotypes and genotypes. We have observed a wide spectrum of disease in a given geographic area: infections in some areas may be extremely aggressive, while those in other areas are indolent or heal quickly. Assuming that in a given locale *M. ulcerans* is genotypically homogeneous, these observations suggest that such host factors as immunity, metabolism, and nutrition play important roles in the natural history of a given infection.

The clinical features of the lesion from which *Mycobacterium* sp. strain 842 was isolated were compatible with the ulcerative granulomatous dermatitis of an *M. marinum* infection. Furthermore, the patient probably acquired his infection in Holland, where he lived for more than 5 years before diagnosis. Although our sequence results are identical to those for the *M. marinum* ITM type, seven phenotypic characteristics differentiated this isolate from *M. marinum* (Table 3). Table 3 also shows a close relationship of *Mycobacterium* sp. strain 842 with some *M. ulcerans* strains from Australia and with *M. shinshuense*. Further biochemical and genetic studies should clarify the relationships between this isolate, *M. marinum*, *M. ulcerans*, and *M. shinshuense*. Imaeda et al. (9) stressed genetic similarities between *M. marinum* and *M. ulcerans* by noting a

high degree of genomic relatedness in DNA-DNA hybridizations. Some phenotypic characteristics are shared by *M. ulcerans* and *M. marinum*, e.g., similar lipid contents (4, 5) and optimal growth at 33°C (28). Although these species are primarily pathogens of the skin, the diseases that they cause differ greatly clinically and epidemiologically (15): *M. ulcerans* causes chronic necrotizing ulcers, largely limited to focal areas of tropical countries, and *M. marinum* provokes cutaneous granulomas, but is worldwide. Further biochemical and genetic studies should clarify the phylogenetic relationship of *M. marinum* and *M. ulcerans.*

A new mycobacterium from an ulcer in the skin of a Japanese girl who had never lived outside of Japan (26) was analyzed by Tsukamura and colleagues (24, 25). Those investigators considered this organism a new species, *M. shinshuense* (24), that is phenotypically related to *M. ulcerans* (25) but that differs biochemically from *M. ulcerans*. Hofer et al. (8) considered a G at position 1248 of the 16S rRNA as being characteristic of *M. ulcerans*. We found a G at position 1248 in *M. shinshuense* (Table 1), suggesting a genetic relationship between *M. ulcerans* and *M. shinshuense. M. shinshuense*, like the Australian, African, and North American types, could therefore be another geographic variant of *M. ulcerans*. Complete sequencing of *M. shinshuense* 16S rRNA would confirm this relatedness.

In conclusion, analysis of the 3′-terminal region of the 16S rRNA sequences of *M. ulcerans*, *M. marinum*, and *M. shinshuense* defined three subgroups of *M. ulcerans* and suggested the potential usefulness of this sequence for the rapid detection of *M. ulcerans*. The subgroup divisions correspond to the differences in the geographic origins of the strains and to some phenotypic properties. We suggest, however, that the extent of this variability be evaluated by analyzing isolates from different foci. This variability should stimulate inquiries into the phylogenetic relatedness among *M. ulcerans*, *M. marinum*, and *M. shinshuense.*

We thank D. Dawson for providing *M. ulcerans* isolates and C. Maeckelbergh and C. Rotteglia for assistance in the preparation of the manuscript.

This work was supported by the Damien Foundation (Brussels), grant 3.4601.90 from the Funds for Medical Scientific Research (Brussels), and the American Registry of Pathology (Washington, D.C.).

REFERENCES

1. **Böddinghaus, B., J. Wolters, W. Heikens, and E. C. Böttger.** 1990. Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. FEMS Microbiol. Lett. **70:**197–204.
2. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.
3. **Christie, M.** 1987. Suspected *Mycobacterium ulcerans* in Kiribati. Med. J. Aust. **146:**600–609.
4. **Daffé, M., M. A. Lanéeile, and C. Lacave.** 1991. Structure and stereochemistry of mycolic acids of *Mycobacterium marinum* and *Mycobacterium ulcerans*. Res. Microbiol. **142:**397–403.
5. **Daffé, M., A. Varnerot, and V. Vincent Lévy-Frébault.** 1992. The phenolic mycoside of *Mycobacterium ulcerans*: structure and taxonomic implications. J. Gen. Microbiol. **138:**131–137.
6. **Flood, P., A. Street, P. O'Brien, and J. Hayman.** 1994. *Mycobacterium ulcerans* infection on Phillip Island, Victoria. Med. J. Aust. **160:**160.
7. **Hayman, J.** 1993. Out of Africa: observations of the histopathology of *Mycobacterium ulcerans* infections. J. Clin. Pathol. **46:**5–9.
8. **Hofer, M., B. Hirschel, P. Kirschner, M. Beghetti, A. Kaelin, C.-A. Siegrist, S. Sutter, A. Teske, and E. C. Böttger.** 1993. Brief report: disseminated osteomyelitis from *Mycobacterium ulcerans* after a snakebite. N. Engl. J. Med. **328:**1007–1009.
9. **Imaeda, T., G. Broslawski, and S. Imaeda.** 1988. Genomic relatedness among mycobacterial species by nonisotopic blot hybridization. Int. J. Syst. Bacteriol. **38:**151–156.
10. **Jackson, K., R. Edwards, D. E. Leslie, and J. Hayman.** 1995. Molecular method for typing *Mycobacterium ulcerans*. J. Clin. Microbiol. **33:**2250–2253.
11. **Johnson, P. D. R., M. G. K. Veitch, P. E. Flood, and J. A. Hayman.** 1995. Mycobacterium infection on Phillip Island, Victoria. Med. J. Aust. **162:**221–222.
12. **Josse, R., A. Guédénon, J. Aguiar, S. Anagonou, C. Zinsou, C. Porst, J. Foundohou, and J. E. Touze.** 1994. L'ulcére de Buruli, une pathologie peu connue au Bénin. A propos de 227 cas. Bull. Soc. Pathol. Exot. **87:**170–175.
13. **MacCallum, P., J. C. Tolhurst, G. Buckle, and H. A. Sissons.** 1948. A new mycobacterial infection in man. I. Clinical aspects. J. Pathol. Bacteriol. **60:**93–101.
14. **Marston, B. J., M. O. Diallo, C. R. Horsburgh, Jr., I. Diomande, M. Z. Saki, J.-M. Kanga, G. Patrice, H. B. Lipman, S. M. Ostroff, and R. Good.** 1995. Emergence of Buruli ulcer disease in the Daloa region of Côte d'Ivoire. Am. J. Trop. Med. Hyg. **52:**219–224.
15. **Meyers, W. M.** 1995. Mycobacterial infections of the skin, p. 291–377. *In* G. Seifert (ed.), Tropical pathology. Springer-Verlag Heidelberg.
16. **Mitchell, P. J., I. V. Jerrett, and K. J. Slee.** 1984. Skin ulcers caused by *Mycobacterium ulcerans* in koalas near Bairnsdale, Australia. Pathology **16:** 256–260.
17. **Pimsler, M., T. A. Sponsler, and W. M. Meyers.** 1988. Immunosuppressive properties of the soluble toxin from *Mycobacterium ulcerans*. J. Infect. Dis. **157:**577–580.
18. **Portaels, F.** 1978. Etude d'Actinomycétales isolées de l'homme et de son environnement en Afrique Centrale. Ph.D. thesis. Faculté des Sciences, Université Libre de Bruxelles, Bruxelles.
19. **Portaels, F.** 1989. Epidémiologie des ulcères à *Mycobacterium ulcerans*. Ann. Soc. Belge Med. Trop. **69:**91–103.
20. **Portaels, F.** 1995. Epidemiology of mycobacterial diseases. Clinics Dermatol. **13:**207–222.
21. **Portaels, F., L. Realini, L. Bauwens, B. Hirschel, W. M. Meyers, and W. De Meurichy.** 1996. Mycobacteriosis caused by *Mycobacterium genavense* in birds kept in a zoo: an 11-year survey. J. Clin. Microbiol. **34:**319–323.
22. **Rogall, T., J. Wolters, T. Flohr, and E. C. Böttger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. Int. J. Syst. Bacteriol. **140:**323–330.
23. **Tsang, A. Y., and E. R. Farber.** 1973. The primary isolation of *Mycobacterium ulcerans*. Am. J. Clin. Pathol. **59:**688–692.
24. **Tsukamura, M.** 1983. Numerical classification of 280 strains of slowly growing mycobacteria. Microbiol. Immunol. **27:**315–334.
25. **Tsukamura, M., K. Kaneda, T. Imaeda, and H. Mikoshiba.** 1989. A taxonomic study on a *Mycobacterium* which caused skin ulcer in a Japanese girl and resembled *Mycobacterium ulcerans*. Kekkaku **64:**691–697.
26. **Tsukamura, M., and H. Mikoshiba.** 1982. A new *Mycobacterium* which caused skin infection. Microbiol. Immunol. **26:**951–955.
27. **Van Keulen, P. H. J.** Personal communication.
28. **Vincent Lévy-Frébault, V., and F. Portaels.** 1992. Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. Int. J. Syst. Bacteriol. **42:**315–323.