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Growth and cytotoxic activity by *Mycobacterium ulcerans* in protein-free media

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Abstract

The pathogenic slow-growing *Mycobacterium ulcerans* has, until now, been cultured in liquid media containing albumin. Here, we report the first description of use of Sauton medium and modified Reid medium, two protein-free media, in which *M. ulcerans* was able to grow and produce its toxin, a major virulence factor of this environmental organism which causes a skin disease commonly called Buruli ulcer. These results suggest that Sauton and modified Reid may be useful for certain fields of *M. ulcerans* research requiring protein-free growth conditions. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Liquid cultivation; Protein-free medium; Toxin activity; *Mycobacterium ulcerans*

1. Introduction

The slow-growing *Mycobacterium ulcerans* is the etiologic agent of Buruli ulcer (BU) [1,2], a human disease primarily of the skin which is considered to be the third most common mycobacteriosis after tuberculosis and leprosy [3]. From the first description of *M. ulcerans* infection by MacCallum [1] until now, our knowledge of the pathogenesis of *M. ulcerans* infection has progressed and has essentially been based on investigations related to the toxin, the major virulence factor of *M. ulcerans* [4–6]. This toxin was recently characterized as a lipid [7] whose chemical structure corresponds to a polyketide [8].

Further aspects of *M. ulcerans* microbiology need to be studied [9]. To date, studies have probably been hampered by the fact that cultivation of this organism remains laborious. Its generation time is approximatively 36 h [10], primary culture may take several months on Löwenstein-Jensen (L-J) medium, in contrast to 3–4 weeks for subcultures [10,11]. Cultivation of *M. ulcerans* for toxin characterization has been performed in media containing albumin: Dubos albumin medium [4–6] and Middlebrook 7H9 medium supplemented with oleic acid,

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albumin, dextrose and catalase [7,8]. Even though the presence of albumin in the media did not prevent isolation of *M. ulcerans* lipid toxin [8], it may however, be a serious handicap for the characterization of other virulence factors of *M. ulcerans*, especially of protein nature. Until now, the use of protein-free media in *M. ulcerans* cultivation has not been described.

We report here that Sauton medium and modified Reid medium allow not only *M. ulcerans* multiplication, but also the production of *M. ulcerans* toxin. Results obtained in this study suggest the convenience of this two media for cultivation of *M. ulcerans* under protein-free conditions.

2. Materials and methods

2.1. Strains and cultivation

Table 1 shows the source of the mycobacterial strains studied. Identification was performed by classical phenotypic characteristics [12] and by PCR and oligonucleotide-specific capture plate hybridization as previously described [11]. Strains ITM 97-96, ITM 97-101, ITM 97-107 and ITM 97-112 were recently isolated from patients from Benin; strains ITM 5146 and ITM 5150 from the Democratic Republic of Congo, had been previously freeze dried. Since the virulence of *M. ulcerans* may decline by subculturing [4,8], the latter two strains were passaged once through mouse footpads, and it was confirmed that ITM 5146 is non-virulent, and was used in the present study as a negative control. All six strains were subcultured on L-J medium. Homogeneous mycobacterial suspensions were made by vortexing with glass beads. Liquid media were inoculated at a final concentration of 1 mg ml^{-1} (wet weight) as indicated by Pattyn [10]. Dubos albumin medium (DAM) was prepared according to manufacturer's (Difco, Detroit, USA) instructions. Sauton medium (SM) and modified Reid medium (MRM) were prepared as described by Landi [13]. For stationary cultures (SC), 100 ml of inoculated media was incubated in 1 l Roux flasks at 33°C. To determine growth curves, each mycobacterial suspension was inoculated into four flasks containing the same medium, and every 2 weeks one of them was harvested to estimate bacterial yield by viable count. For orbital-shaking cultures (OSC), 100 ml of media supplemented with 0.05% (v/v) Tween 80 was inoculated and incubated at 33°C in 1 l roller bottles gently rotating at 2 r.p.m. (Lab-Line, Melrose Pank In, IL, USA). For bacterial viable count determination, cultures were first centrifugated ($3000 \times g$ for 30 min at 4°C) and then bacterial cells were declumped by vortexing with glass beads before resuspension in the original culture volume with phosphate buffered saline (PBS) containing 0.05% (v/v) of Tween 80. Serial 10-fold dilutions were made and 0.1 ml aliquots were cultured in triplicate on L-J slants. Colony forming units (CFU) were counted after 6 weeks of incubation at 33°C. Results shown are representatives of three separate experiments.

2.2. Preparation of culture filtrates and toxin activity assays

Eight week old cultures were centrifugated as indicated above, and supernatants were filter sterilized (Sterivex-GP 0.22 μm filter, Millipore Corporation, Bedford, USA) for obtaining culture filtrates. Four ml aliquot from each sterile supernatant was directly dialyzed against PBS, and designed unconcentrated culture filtrate (UCF). The remainder was concentrated (CCF) by ultrafiltration (Amicon PM10 membrane, Beverly, USA), and the volume was adjusted to 4 ml before dialysis. For toxin activity assays, murine fibroblast L929 cell line (purchased from American Type Culture Collection, Rockville, MD, USA) was maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Gibco BRL) and 0.1 mg ml^{-1} of streptomycin and 100 U ml^{-1} of penicillin. Cells were plated in 96-well tissue culture plates (Corning Incorporated, Corning, NY, USA) at 5×10^4 cells per well in 0.1 ml of the above medium to allow cell adherence. After 24 h, the medium was removed. Sterile dialyzed preparations of culture filtrates were mixed with equal volume of this medium, and 0.1 ml was added to L929 cells in four wells per sample. After 48 h of incubation at 37°C in 5% CO_2, cells were examined microscopically for rounded up and detached cells as previously described [7]. Results are representatives of three separate experiments.

3. Results and discussion

In order to investigate whether *M. ulcerans* can grow in protein-free media, growth curves of *M. ulcerans* strain ITM 97-112 were determined in two protein-free media (SM and MRM), and in DAM. Fig. 1 shows that logarithmic multiplication was nearly identical in the three media during the first 2 weeks of growth, and then the growth in protein-free media (SM and MRM) increased slightly until the stationary phase was reached after 6 weeks of incubation. In contrast in DAM, a decline phase appeared at 4 weeks. These results demonstrate an improved growth of *M. ulcerans* in SM and MRM. It is possible that the use of fresh inocula of organisms harvested from 3–4 weeks cultured on L-J medium may have enhanced growth in this study. Pattyn [10] has shown that the inoculation of large quantities of *M. ulcerans* bacilli in the logarithmic phase enhances growth, and suggested that the long incubation time necessary for primary cultures from patient biopsies was due to the small proportion of viable bacilli in tissues.

M. ulcerans is described to clump and form large cell aggregates in liquid cultivation [1,10] and this tendency may provoke inhibition of growth. To study the influence of Tween-associated shaking conditions on *M. ulcerans* growth, three *M. ulcerans* strains were cultured in DAM and MRM. Fig. 2 shows that bacterial yield was increased under OSC 6-log in DAM and 2-log in MRM, compared with SC. Although we have recently shown that low oxygen tension enhances *M. ulcerans* growth in Bactec culture system [14], we propose that the fact of this improvement of bacterial yield observed here in large scale cultivation probably accounts for the declumping effect of both an orbital shaker and the presence of Tween 80 in media [15], and should further be investigated in the Bactec culture system.

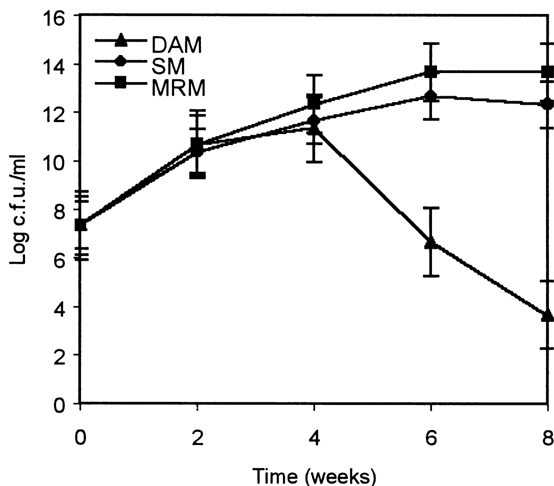

Fig. 1. Growth of *M. ulcerans* ITM 97-112 in SM, MRM and DAM. Log CFU ml^{-1} estimated every 2 weeks of growth period, is shown as a function of time. Mean values and standard deviations are shown from three separate experiments.

Table 1
List of *M. ulcerans* strains and their origin

Strain number[a]	Origin	Year of isolation
ITM 5146	Democratic Republic of Congo	1963
ITM 5150	Democratic Republic of Congo	1962
ITM 97-96	Benin	1997
ITM 97-101	Benin	1997
ITM 97-107	Benin	1997
ITM 97-112	Benin	1997

[a]ITM, Institute of Tropical Medicine.

M. ulcerans toxin has been described as causing cytoskeletal rearrangement and cell division arrest in G1 phase of cell cycle [7,8]. In order to determine whether protein-free media allow production of *M. ulcerans* toxin, preparations of culture filtrates from 8 week old cultures were tested for toxin activity against L929 cell line. Table 2 shows that *M. ulcerans* strain ITM 97-112 produced the toxin not only

Table 2
Detection of toxin activity produced by *M. ulcerans* ITM 97-112 in culture media

Bacterial culture media	Toxin activity in[a]	
	UCF	CCF
DAM non-inoculated	−	−
DAM growth-associated	+	+
SM non-inoculated	−	−
SM growth-associated	−	+
MRM non-inoculated	−	−
MRM growth-associated	−	+

Results shown are representatives of three separate experiments.
[a]Toxin activity was assessed on L929 cells with UCF and CCF prepared from 8 week old cultures performed in DAM, SM and MRM under stationary conditions at 33°C.

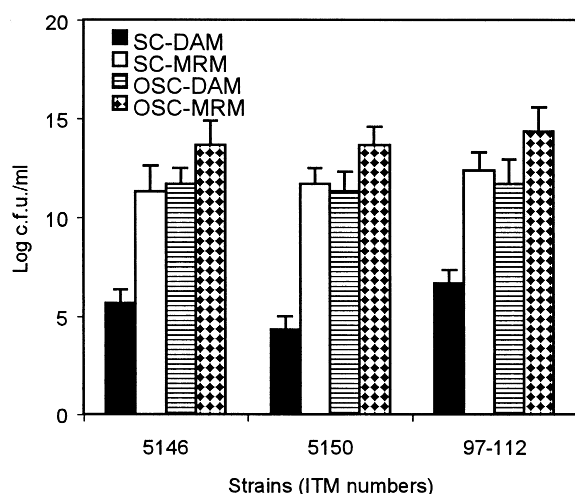

Fig. 2. Effect of orbital-shaking cultures on *M. ulcerans* bacterial yield. Bacterial yield is shown here as log CFU ml^{-1} estimated after 6 weeks of cultivation in DAM and MRM under two types of incubation: SC and OSC. Mean values and standard deviations are shown from three separate experiments.

in DAM as previously described [4–6] but also in protein-free media (SM and MRM). Furthermore we noticed that no toxin activity was observed in UCF from protein-free media compared with UCF from DAM, and suggest that since *M. ulcerans* grown in protein-free media could produce less toxin, it may be necessary to concentrate culture filtrates in order to demonstrate its activity in protein-free media.

We further screened for toxin activity several *M. ulcerans* strains by using the protein-free MRM. Table 3 shows that all recently isolated strains exhibited toxin activity, in contrast to strain ITM 5146, whose

Table 3
Screening for toxin-producing strains in MRM

M. ulcerans strains	Toxin activity[a]
ITM 5146	–
ITM 5150	+
ITM 97-96	+
ITM 97-101	+
ITM 97-107	+
ITM 97-112	+

[a]Toxin activity was assessed on L929 cells with CCF prepared from 8 week old cultures performed in MRM under stationary conditions at 33°C. Results shown are representatives of three separate experiments.

avirulence feature was already demonstrated in the mouse footpad model. These results show that protein-free media especially MRM, may help to identify toxin-producing strains in *M. ulcerans*.

In conclusion we have demonstrated the cultivation of *M. ulcerans* in two protein-free media: Sauton medium and modified Reid medium which preserve the production of toxin activity. This finding may help in some applications needing protein-free cultivation such as the purification and characterization of protein virulence factors of *M. ulcerans*.

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