Experimental pieces of evidence for *Mycobacterium ulcerans* dormancy.

Running title: *Mycobacterium ulcerans* dormancy.

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ABSTRACT

Background: Whether *Mycobacterium ulcerans*, the etiological agent of the neglected Buruli ulcer in numerous tropical countries, would exist in a dormant state as reported for closely related *Mycobacterium* species, is not established.

Methodology: Six *M. ulcerans* strains were exposed to a progressive depletion in oxygen for two months, using a previously described Wayne model of dormancy; and further examined by microscopy using DDD staining, microcalorimetry and subculture in the presence of dead and replicative *M. ulcerans* as controls.

Principal Findings/Conclusions: *M. ulcerans* CU001 strain died during the progressive oxygen depletion and four of five remaining strains exhibited Nile Red-stained intracellular lipid droplets after DDD staining and a 14-20-day regrowth when exposed to ambient air, diagnosing dormancy. A fifth *M. ulcerans* 19423 strain stained negative in DDD and slowly regrew in 27 days. Three tested *M. ulcerans* strains yielded microcalorimetric pattern similar to that of the negative (dead) homologous controls, differing from that of the homologous positive (replicative) controls. The relevance of these experimental observations, suggesting a previously unreported dormancy state of *M. ulcerans*, needs to be investigated in the natural ecological niches where *M. ulcerans* thrive and in Buruli ulcer lesions.

43 **Author summary:**

44 *Mycobacterium ulcerans* is an environmental opportunistic pathogen of mammals
 45 and humans, causing a subcutaneous necrotizing infection named Buruli ulcer.
 46 Molecular detection of *M. ulcerans* DNA revealed different ecological niches where
 47 *M. ulcerans* may thrive, but the molecular biology approach does not catch the
 48 physiological state of *M. ulcerans* in these different ecological niches. Thus, the
 49 reservoir and the mode of transmission of *M. ulcerans* remain elusive. Here, we
 50 investigated experimental dormancy of *M. ulcerans* by using a previously described
 51 Wayne model of dormancy coupled with microscopy using DDD staining,
 52 microcalorimetry and subculture. Our findings demonstrate for the first time that
 53 some *M. ulcerans* strains exhibit a physiological state of dormancy; potentially
 54 limiting isolation and culture of *M. ulcerans* from environmental niches.

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INTRODUCTION

Mycobacterium ulcerans is an environmental non-tuberculous mycobacterium acting as an opportunistic pathogen of mammals and humans in whom it is responsible for a subcutaneous necrotizing infection named Buruli ulcer [1]. Buruli ulcer lesions are primarily caused by cytotoxic macrolide exotoxins named mycolactones, naturally secreted by *M. ulcerans*, of which seven different natural variants have been characterized in the several genotypes of *M. ulcerans* clinical isolates [2, 3]. All these variants target the Wiskott-Aldrich protein Arp2/3 in targeted cells, yet exhibiting various degrees of cytotoxicity, *in vitro* [4, 5]. These observations all issued from the investigations of *M. ulcerans* clinical isolates as no isolate has been made from the environmental sources where this opportunistic pathogen is regularly detected, except from one firmly documented *M. ulcerans* isolate made from one *Gerris* sp. aquatic insect from a Buruli ulcer-endemic area in Benin, West Africa [6]; contrasting with numerous *M. ulcerans* isolates made from clinical sources in Buruli ulcer patients and some mammals [7, 8].

The sharp discrepancy in the yield of *M. ulcerans* recovered from clinical sources versus environmental sources has been attributed to the intrinsic fastidiousness of the pathogen which is requiring a narrow 28-30°C growth temperature and specific culture medium; and to the overgrowth of reportedly contaminating bacteria and fungi [9]. Yet, none of these two limitations really differentiate clinical from environmental samples so that the exact reason for the tremendous difficulties in isolating environmental *M. ulcerans* remained largely unknown.

We herein explored experimentally a third line of explanation by investigating the possibility that *M. ulcerans* may exhibit a dormant state, less prone to laboratory

cultivability than the dynamic state encountered in clinical samples. We indeed report that *M. ulcerans* may behave as a dormant mycobacterium and we showed that it is possible to convert the dormant into a dynamic state, opening a new venue for the isolation of still unexplored, environmental *M. ulcerans*.

MATERIALS AND METHODS

Wayne model of dormancy for *M. ulcerans*. Six different strains of *M. ulcerans* including *M. ulcerans* CU001, *M. ulcerans* ATCC 19423, *M. ulcerans* ATCC 33728, *M. ulcerans* ATCC 25900, *M. ulcerans* ATCC 25894 and *M. ulcerans* ATCC 25898 were investigated in this work. These strains are representative of the 02 genomic lineages currently described in *M. ulcerans* [10]; and originated from Ghana, Australia, Japan, USA, Ouganda, Benin, respectively [11]. These six *M. ulcerans* strains were cultured in Middlebrook 7H9 broth supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC) (Becton Dickinson, Sparks, MD, USA) and 0.5% glycerol at 30°C in a shaking incubator. One non-inoculated tube was run in parallel as a negative control tube. These tubes were continuously incubated in the same conditions without changing the medium. After 50-day incubation of these tubes in this condition, the optical density (OD) of 0.2- 0.3 at 600 nm., a 100-μL aliquot of each culture was suspended in one Hungate tube containing of 10 mL of Dubos broth (Becton Dickinson) in the presence of 1.5 μg/mL methylene blue (Sigma-Aldrich, Saint-Quentin-Fancy, France) as an indicator of oxygen depletion as previously described [12]. This Wayne model of dormancy initially described for *Mycobacterium tuberculosis* H37Rv relies on the gradual depletion of oxygen [13]. Accordingly, Hungate tubes were incubated at 30°C for up to 60 days with continuous shaking, with a weekly visual inspection (Fig. 1).

DDD staining. After two-month incubation of the six *M. ulcerans* strains in the Hungate tubes in anaerobic conditions, a 20 µL-volume of each suspension was smeared on glass slide, air dried and stained using DDD staining protocol as previously described [12,13]. DDD staining was also applied on the aerobic and heat inactivated cultures of the six *M. ulcerans* strains. Microscopic observation was performed using a 63X/1.4NA oil immersion objective of Zeiss Spinning Disk Confocal microscopy driven by Zen software (Zeiss, Marly-le-Roi, France).

Microcalorimetry test. Isothermal microcalorimetry was used to evaluate heat production by three *M. ulcerans* strains (*M. ulcerans* 33728, *M. ulcerans* 25900 and *M. ulcerans* 25894 strains) using a CalScreener prototype microcalorimeter (Symcel, Solna, Sweden). Briefly, 10⁶ colony forming units (CFUs) / mL of Middlebrook 7H9 medium supplemented with 10% OADC of dynamic mycobacteria of each one of the three investigated strains, were incubated for 1h at 100°C and further sonicated at 37 kHz for 30 minutes in order to get so-called dead mycobacteria. Then, 10⁶ CFUs / mL dead and 10⁶ CFUs / mL dynamic mycobacteria (in Middlebrook medium) and 10⁶ CFUs / mL dormant mycobacteria (in Dubos medium) were incubated at 30°C for 17 hours before being prepared for microcalorimetry. For microcalorimetry, 200 µL of each suspension were inoculated into 400-µL titanium tubes in triplicate, 09 tubes containing 200 µL of Middlebrook 7H9 medium supplemented with 10% OADC and 06 tubes containing 200 µL of Dubos broth were used as negative controls. These 24 sealed titanium tubes were placed in the microcalorimeter previously equilibrated at 30°C for 4 days. Individual heat production rates (i.e., $W = J/s$ = heat flow or thermal power) of each titanium tube was measured continuously in real time during 100 hours as previously described [14].

Subculture of dormant *M. ulcerans* strains in aerobic conditions. After the six *M. ulcerans* strains here investigated have been incubated for 60 days in anaerobic conditions in DUBOS medium, 100 µL were collected using 30-Gauge syringes to avoid oxygen penetration and centrifuged at 3,000 g for 15 min. The pellet was then seeded on Middlebrook 7H10 agar plates (Becton Dickinson) incubated at 30°C for 27 days in aerobic conditions. An identification of the cultured bacteria was carried out by molecular biology using real-time PCR targeting the ketoreductase b (kr-b) gene as previously described [15].

RESULTS

Visual inspection of tubes. After 24 to 44-day incubation at 30°C, all the *M. ulcerans*-inoculated tubes decolorized and a bacterial pellet appeared at the bottom except for three tubes inoculated with *M. ulcerans* ATCC CU001 strain; whereas the negative control tube remained blue-colored without any visible pellet (Fig. S1). All colonies were confirmed to be *M. ulcerans* (Supplementary Table).

DDD staining result. After 2-month incubation in anaerobic conditions, DDD staining demonstrated the presence of red fluorescent-intracellular inclusions in four *M. ulcerans* strains except for *M. ulcerans* ATCC CU001 and *M. ulcerans* ATCC 19423 (Fig. 1, Fig. S2). These Nile red-positive lipidic bodies were previously reported as *M. tuberculosis*-dormancy biomarkers [12]. The metabolic activity marker fluorescein diacetate stained *M. ulcerans* mycobacteria either green fluorescent or not (Fig. 1, Fig. S2) [12]. However, in all the tubes incubated under ambient air, all the 6 *M. ulcerans* strains stained positively with fluorescein diacetate positive whereas and no Nile red-positive bodies were observed.

Microcalorimetry test results.

After 100 hours of heat-flow measurements by the microcalorimeter instrument, we observed a heat-flow production by dynamic *M. ulcerans* 33728, *M. ulcerans* 25894 and *M. ulcerans* 25900 strains (Fig. 2). The dynamic *M. ulcerans* 25900 strain reached a heat-flow peak of $\approx 2.83 \mu\text{W}$ after 36-hour incubation, while a heat-flow peak of $\approx 3.89 \mu\text{W}$ for *M. ulcerans* 33728 strain and $\approx 1.84 \mu\text{W}$ for *M. ulcerans* 25894 strain was reached after 92-hour incubation and 94-hour incubation, respectively. Dead mycobacteria used as negative controls produced no heat-flow during all the period of microcalorimetry experiment for any the three investigated strains (Fig. 2). Apart from the dormant *M. ulcerans* 33728 strain and the dormant *M. ulcerans* 25900 strain for which no heat-flow has been detected by microcalorimetry test, interestingly, one of the triplicate dormant *M. ulcerans* 25894 strain produced a heat-flow that reached the peak of $4.06 \mu\text{W}$ after ≈ 77 hours.

Subculture of dormant *M. ulcerans* strains.

Two-month-old anaerobic cultures plated on Middlebrook 7H10 agar medium, grew colonies after 14-27-day incubation at 30°C under ambient air, except for *M. ulcerans* ATCC CU001 which never regrew after 6-week observation.

DISCUSSION

Experimental data here reported indicated that *M. ulcerans* was able to sustain viability two months after progressive depletion in oxygen, with criteria for dormancy; a previously unreported property of the species *M. ulcerans*. These experimental data have been validated by the negativity of the negative controls along with congruent conclusions achieved by three experimental approaches. With

respect to its phylogenetic position among mycobacteria, the fact that *M. ulcerans* exhibited dormancy was not entirely surprising: dormancy has already been reported for *Mycobacterium tuberculosis* [13], *Mycobacterium bovis* BCG [16], *Mycobacterium smegmatis* [17] and *Mycobacterium avium* subspecies *paratuberculosis* [18]. In this study, microcalorimetry was used for the first time to investigate the physiology of *M. ulcerans*. Indeed, microcalorimetry had been previously used in determining the minimum inhibitory concentration of isoniazid, ethambutol, and moxifloxacin against *M. smegmatis*, *M. avium*, and *M. tuberculosis* [19]; but never incorporated *M. ulcerans*, to our knowledge. Here, microcalorimetry yielded data aligned with those achieved using the DDD staining as well as culture; and indicated that metabolism of dormant *M. ulcerans* was reduced to the point that heat flow curves of dormant *M. ulcerans* were similar to those of dead *M. ulcerans*; except that one dormant *M. ulcerans* 25894 strain did produce detectable increasing heat, firmly indicated that this strain was not dead, but indeed viable and dormant.

A second, unanticipated observation was that the extent of dormancy was dependent on the *M. ulcerans* strain. In fact, one strain *M. ulcerans* CU001 died during progressive depletion in oxygen, and we have no explanation for this observation. In particular, methylene blue here used to monitor oxygen depletion in the tubes, has been used here at a concentration 100 times below the minimal inhibitory concentration we previously reported for this particular strain [20]. This observation was unexpected because strain-dependent dormancy was not previously reported for any other investigated mycobacteria.

Indeed, 38 of 48 genes reported in the dormancy operon DosR in *M. tuberculosis*, are retrievable *M. ulcerans* genomes we previously examined [21], in

205 particular, the key regulator gene *dosR* was detected in these *M. ulcerans* genomes
206 [21].

207 Whether *M. ulcerans* dormancy could be of clinical interest beyond the
208 environmental aspect remains to be investigated as dormancy correlates with an
209 increased resistance to otherwise effective antimycobacterial drugs, in the case of *M.*
210 *tuberculosis* [16]. The DDD staining here applied to *M. ulcerans*, offers a convenient
211 tool to further explore the clinical relevance of the experimental observations here
212 reported.

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Figure legends.

Fig. 1 DDD staining of *M. ulcerans* ATCC 33728. A) Dormant *M. ulcerans* ATCC 33728 incubated for two months using the Wayne model of dormancy B) Dynamic *M. ulcerans* ATCC 33728 incubated in ambient atmosphere. C) Dead, heat-inactivated *M. ulcerans* ATCC 33728. Microscopic observations were performed using a 63X/1.4NA oil immersion objective of Zeiss spinning disk.

Fig. 2 Heat-flow produced by three different *M. ulcerans* strains, according to their biological state **(A)** *M. ulcerans* 33728 strain; **(B)** *M. ulcerans* 25894 strain; **(C)** *M. ulcerans* 25900 strain. Green curves: dynamic mycobacteria; orange curves: dormant mycobacteria; red curves: dead mycobacteria.

Supplementary figures.

Fig. S1. Hungate tubes containing six *M. ulcerans* strains at day 0 and after 27-day incubation in progressive anaerobic atmosphere according to Wayne dormancy model. This panel features changing color of the culture medium, from blue to transparency, indicative of oxygen depletion.

Fig. S2. DDD staining on Wayne model of dormancy after 02-month incubation of *M. ulcerans* CU001, *M. ulcerans* ATCC 19423, *M. ulcerans* ATCC 25900, *M. ulcerans* ATCC 25894 and *M. ulcerans* ATCC 25898. Microscopic observation was performed using a 63X/1.4NA oil immersion objective of Zeiss Spinning Disk.

315 **Supplementary Table.** Real-time PCR cycle threshold (CT) values for *M. ulcerans*
 316 strains sub cultured after dormancy.

Strain	CT value
<i>M. ulcerans</i> strain 25900	25.35
<i>M. ulcerans</i> strain 33728	25.22
<i>M. ulcerans</i> strain 25894	24.36
<i>M. ulcerans</i> strain CU001	22.32
<i>M. ulcerans</i> strain 19423	29.06
<i>M. ulcerans</i> strain 25898	24.71

317

Figure

Figure 2.

A.

B.

C.

Figure