Impact of Temperature and Oxygen Availability on Gene Expression Patterns of *Mycobacterium ulcerans*

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Abstract

Buruli ulcer disease is a neglected tropical disease caused by the environmental pathogen *Mycobacterium ulcerans*. The *M. ulcerans* major virulence factor is mycolactone, a lipid cytotoxic compound whose genes are encoded on plasmid. Although an exact reservoir and mode(s) of transmission is unknown, data provide evidence of both. First, Buruli ulcer incidence and *M. ulcerans* presence have been linked to slow-moving water with low oxygen.

M. ulcerans has also been suggested to be sensitive to UV due to termination in *crtI,* encoding a phytoene dehydrogenase, required for carotenoid production. Further, *M. ulcerans* has been shown to cause disease following puncture, but not when introduced to open abrasion sites, suggesting puncture necessary for transmission and pathology. Despite these findings, the function and modulation of mycolactone and other genes in response to dynamic abiotic conditions such as UV, temperature, and oxygen, has not been shown. In this study, we investigated modulation of mycolactone and other genes on exposure to changing UV and oxygen microenvironmental conditions. Mycolactone expression was downregulated on exposure to single stress high temperature and did not change significantly with exposure to UV, however, was upregulated when exposed to microaerophilic conditions. Mycolactone expression was downregulated under combined stress of high temperature and low oxygen but there was upregulation of several stress response genes. Taken together, results suggest that temperature shapes *M. ulcerans* metabolic response more so than UV exposure or oxygen requirements. These data aid to define the environmental niche of *M. ulcerans* and metabolic responses during initial human infection.

Importance

Buruli ulcer is a debilitating skin disease caused by the environmental pathogen, *Mycobacterium ulcerans*. *M. ulcerans* produces a toxic compound, mycolactone that leads to tissue necrosis and ulceration. Barriers to preventing Buruli ulcer include an incomplete understanding of *M. ulcerans* reservoirs, how the pathogen is transmitted, and under what circumstances mycolactone and other *M. ulcerans* genes are expressed and produced in its natural environment and in the host. We conducted a study to investigate *M. ulcerans* gene expression under several individual or combined abiotic conditions. Our data showed that mycolactone expression was downregulated under combined stress of high temperature and low oxygen but there was upregulation of several stress response genes. These data are among only a few studies measuring modulation of mycolactone and other *M. ulcerans* genes that could be involved in pathogen fitness in its natural environment and virulence while within the host.

Keywords: Buruli ulcer; *Mycobacterium ulcerans*; Mycolactone; gene expression

Introduction

Buruli ulcer is a neglected tropical disease caused by an environmental pathogen, *Mycobacterium ulcerans*. The disease is characterized by an often painless nodule that can later develop into an ulcer (1). Buruli ulcer is the third most common mycobacterial infection and has been reported in over 33 countries worldwide (1). Buruli ulcer is often associated with functional limitations and limb deformities in cases of deferred treatment, and imposes a significantly negative medical, psychological and socio-economic impact on affected patients (2, 3). The major virulence factor of *M. ulcerans* is mycolactone, a lipid cytotoxic compound whose genes are encoded on the large plasmid, pMUM001(4). Mycolactone diffuses through healthy tissue leading to pathology that impacts a wide variety of cells through modulation of immune functioning, inhibiting immune regulating proteins, or by causing cytoskeletal rearrangement, cell cycle arrest, necrosis or apoptosis, depending on cell type (5).

Morbidity has, in part, been exacerbated by the fact that the mode of *M. ulcerans* transmission remains unknown; however, multiple mechanisms have been proposed and investigated (6, 7). Epidemiological evidence links Buruli ulcer incidence to slow moving aquatic habitats (8-10). This has also been supported by the finding of *M. ulcerans* DNA in association with aquatic plant biofilms, water filtrand, soil, and invertebrates (11-15). Slow-moving water with low oxygen concentration has also been included as a risk factor (9, 10, 15-17). Laboratory studies showed *M. ulcerans* leads to disease pathology following puncture, but not when introduced to an open abrasion site (18, 19), and mosquitoes and other biting insects have also been implicated as potential mechanical vectors (7).

As an environmental pathogen, *M. ulcerans* naturally resides in a complex community of aquatic life presumably structured by biological interactions and abiotic environmental factors (6). When reviewing other systems, such as the environmental pathogens *Burkholderia cenocepacia* and *Vibrio vulnificus*, low oxygen concentration and high temperature have been shown to upregulate virulence factors (20, 21). Additionally, temperature-regulated toxin production has been observed in pathogenic bacteria such as enterohemorrhagic *Escherichia coli* (EHEC), *Yersinia enterocolitica* and *Bacillus anthracis* (22). Data from these other pathogens studies suggest a need to investigate the possible role of higher temperature and lower oxygen yielding changes in mycolactone production, in establishment of disease and fitness within its natural environment (19). Understanding modulation of mycolactone and other gene expression in response to temperature and oxygen gradients will not only aid in understanding *M. ulcerans* response to changing abiotic conditions in aquatic environments, but also in understanding *M. ulcerans* virulence and pathogenesis leading to disease, depending upon transmission route.

M. ulcerans is also suggested to be UV sensitive due to termination in *crtL,* a gene responsible for carotenoid production (23). As a replicative reservoir of *M. ulcerans* is unknown within aquatic habitats, it is not clear whether *M. ulcerans* replicates in areas that are protected from UV or has developed machineries to counteract adverse UV effects (6). Thus, investigating whether mycolactone influences protection against UV through a pigment mediated or other mechanism can provide further insight into *M. ulcerans* reservoirs.

Few studies have been published exploring mycolactone gene expression. One study showed that transcription of several key mycolactone biosynthetic genes are driven by a SigA-like promoter (24); however, the study did not determine environmental or growth-phase signals inducing mycolactone gene expression. An *in vitro* study showed mycolactone genes downregulated in response to various sugar sources (25), and other research showed that nutrient availability (chitin vs. calcium) regulates several metabolic pathways in *M. ulcerans*; however, mycolactone toxin was not expressed in these nutrient abundant environments suggesting that its expression may be regulated mainly during stressful conditions (26).

In this study, we investigated how exposure to single and combined abiotic factors affected *M. ulcerans* growth, modulation of mycolactone expression via RT-qPCR, and global gene expression of a subset of samples via RNASeq. First, we tested the effect of UV exposure on *M. ulcerans* growth and mycolactone gene expression, then whether growth and mycolactone and other *M. ulcerans* genes were modulated under changing temperature and oxygen, and the combined interactive effect of the latter two. These data fill gaps in knowledge regarding *M. ulcerans* stress response, providing molecular evidence for how fitness may be influenced by abiotic conditions of aquatic ecosystems and in Buruli ulcer wounds.

Results

Little effect of UV exposure on M. ulcerans growth and ER gene expression

Compared to controls no significant difference in *M. ulcerans* growth was observed following exposure to UV for 30 seconds (p=0.18), 2 minutes (p=0.45) or 10 minutes (p=0.10) (Figure

1A). There was a negligible effect on mycolactone gene expression upon exposure of *M. ulcerans* to UV for 30 seconds, whereas there was a slight downregulation with exposure to UV for 2 minutes; but neither of these effects were statistically significant (Figure 1B). However, ER gene expression trended toward significant downregulation on exposure to UV for 10 minutes (p=0.051, Figure 1B).

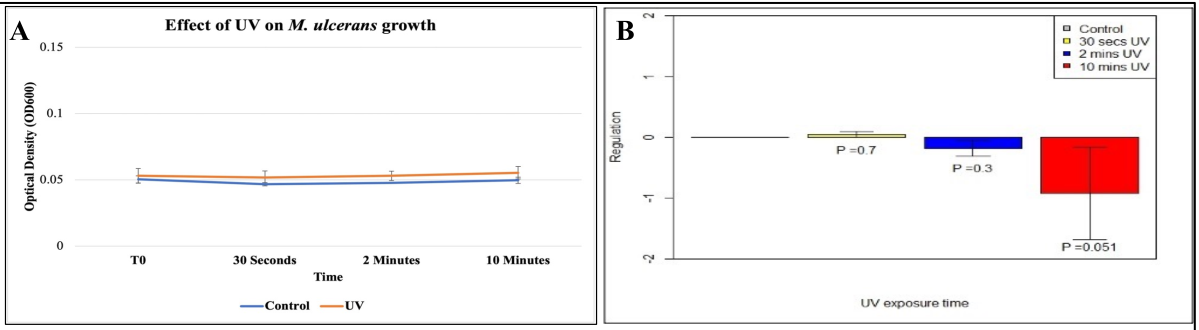

Figure 1. Effects of increasing UV exposure on *M. ulcerans* growth (A) and ER expression (B).

***M. ulcerans* growth and mycolactone expression when exposed to single and combined environmental stressors**

Across all time points there was no statistical difference in *M. ulcerans* growth (at 30^0 C) under microaerophilic or anaerobic conditions in comparison to aerobic conditions (Figure 2A). Similarly, there was no significant difference in *M. ulcerans* growth when subjected to different temperatures (30°C vs 37°C) for 24 hours (Figure 2B). When cultures were brought back to 30°C, those that had been subjected to 37°C were not significantly different from those subjected to 30°C based on growth (Figure 2B).

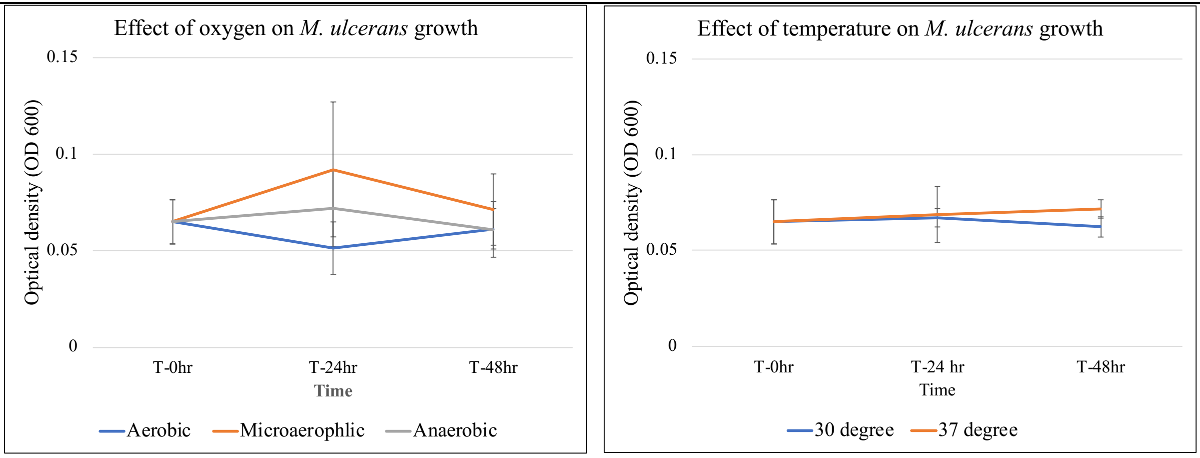

Figure 2. Effect of Oxygen (A) or temperature (B) on *M. ulcerans* growth. (A) Optical density of *M. ulcerans* when exposed to aerobic (blue line), microaerophilic (orange) and anaerobic (grey line) conditions at 30^0C. Exponential *M. ulcerans* initially under aerobic conditions were exposed to their respective oxygen condition for 24 hours and then transferred back to aerobic conditions for an additional 24 hours (48 hours from initial time point). (B) Optical density of *M. ulcerans* when exposed to 30^0C (blue line) and 37^0C (orange line). Exponential *M. ulcerans* initially at 30^0C were exposed to their respective temperature conditions for 24 hours and then exposed back to 30^0C for an additional 24 hours (48 hours from initial time point).

Exposure of *M. ulcerans* to microaerophilic conditions for 24 hours showed significant upregulation of ER gene expression (p=0.0009). Gene expression was slightly downregulated when *M. ulcerans* exposed to microaerophilic conditions at 30^0 C were transferred back to aerobic conditions, but the difference was not statistically significant (Figure 3). *M. ulcerans* exposed to anaerobic conditions for 24 hours had non-significant upregulation of mycolactone gene expression. However, upon transferring the anaerobic exposed bacteria back to aerobic conditions, there was significant (p=0.005) upregulation of mycolactone gene expression compared to controls grown under aerobic conditions over the 3 day study (Figure 3).

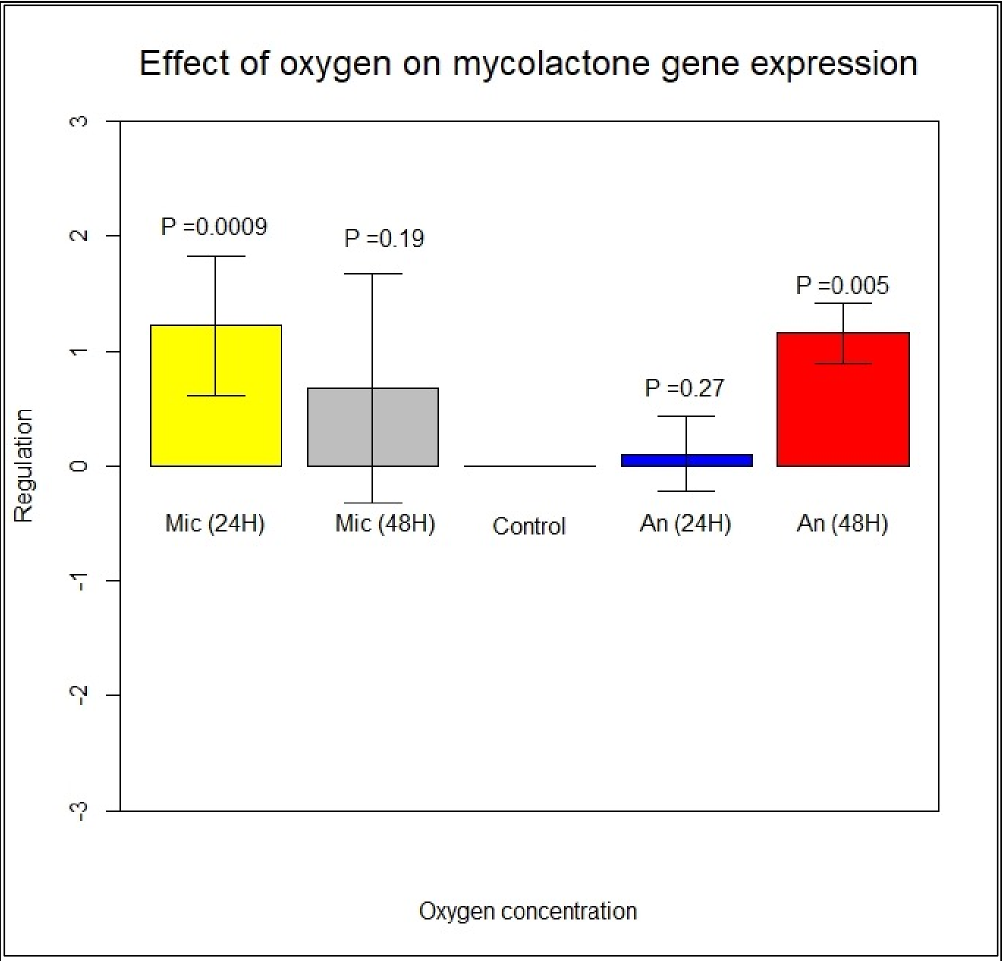

Figure 3. *M. ulcerans* ER regulation after exposure to microaerophilic or anaerobic conditions compared to aerobic conditions. Exposure to a microaerophilic environment for 24 hours caused significant upregulation [Mic (24H); P=0.0009] of ER expression (yellow bar) and transferring the bacteria back to aerobic condition led to slight downregulation in ER expression [Mic (48H), grey bar]. Exposure of *M. ulcerans* to anaerobic conditions for 24 hours led to slight ER gene upregulation [AN (24H), blue bar], but transfer back to aerobic conditions caused significant ER upregulation [AN (48H), P=0.005, red bar] compared to control *M. ulcerans* exposed to aerobic conditions during the entire 3-day experiment (Control bar). Error bars indicate Standard errors. *M. ulcerans* was at 30º C for all the oxygen conditions.

ER gene expression was significantly downregulated when *M. ulcerans* grown aerobically at 30°C (Control) were exposed aerobically at 37°C (p= 0.02923) for 24 hours, and significantly upregulated when brought back to aerobic at 30°C (p=0.0002 compared to control and p=0.001 compared to 37°C at aerobic condition-day 2). Although ER gene expression was downregulated when exposed to 37°C under microaerophilic conditions and upregulated when brought back to 30°C and aerobic conditions, the regulation was not statistically significant (Figure 4A).

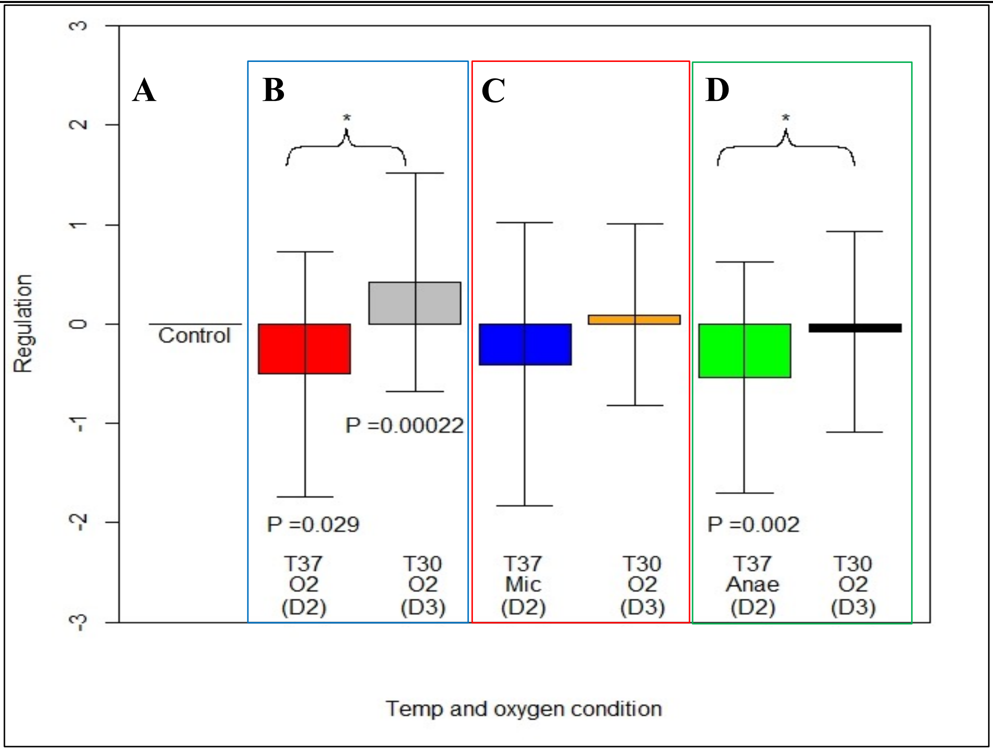

Figure 4. Regulation of ER expression after *M. ulcerans* exposure to differing temperature and oxygen conditions. (A) Control conditions of *M. ulcerans* at 30^{0}C and aerobic conditions from days 1-3; (B and in blue box): *M. ulcerans* exposed to 37^{0}C and aerobic conditions on Day 2 [T37-O2 (D2)] but transferred back to 30^{0}C and aerobic condition on day 3 [T30-O2 (D3)]; (C and in red box): *M. ulcerans* exposed to 37^{0}C and microaerophilic condition on day 2 [T37-Mic (D2)] but transferred back to 30^{0}C and aerobic condition on day 3 [T30-O2 (D3)]; (D and in green box): *M. ulcerans* exposed to 37ºC and anaerobic condition on day 2 [T37-Anae (D2)] but transferred back to 30ºC and aerobic condition on day 3 [T37-O2 (D3)]: Bars indicate standard error. P values indicate significance values between treatment and control. Stars indicate significance within treatments.

The shift of *M. ulcerans* at 30°C under aerobic conditions to 37°C and anaerobic conditions showed significant downregulation of ER gene expression (p=0.002). When the cultures were brought back to control conditions, ER gene expression was not statistically different from the control, but significantly upregulated (p=0.01) compared to *M. ulcerans* at 37°C under anaerobic conditions on day 2 (Figure 4B).

M. ulcerans global stress response on exposure to high temperature and low oxygen conditions

RNAseq analysis was performed to determine regulated gene response to exponentially grown *M. ulcerans* at 30°C under aerobic conditions that were exposed to 37°C or to the combination of 37°C and microaerophilic conditions. A heat-map representing 50 of the top significant differentially regulated genes across treatments and timepoints compared to the control condition is shown in Figure 5, however, a heat map and list of all significantly differentially regulated genes can be found in Supplemental Figure 1 and Supplemental Table 1, respectively.

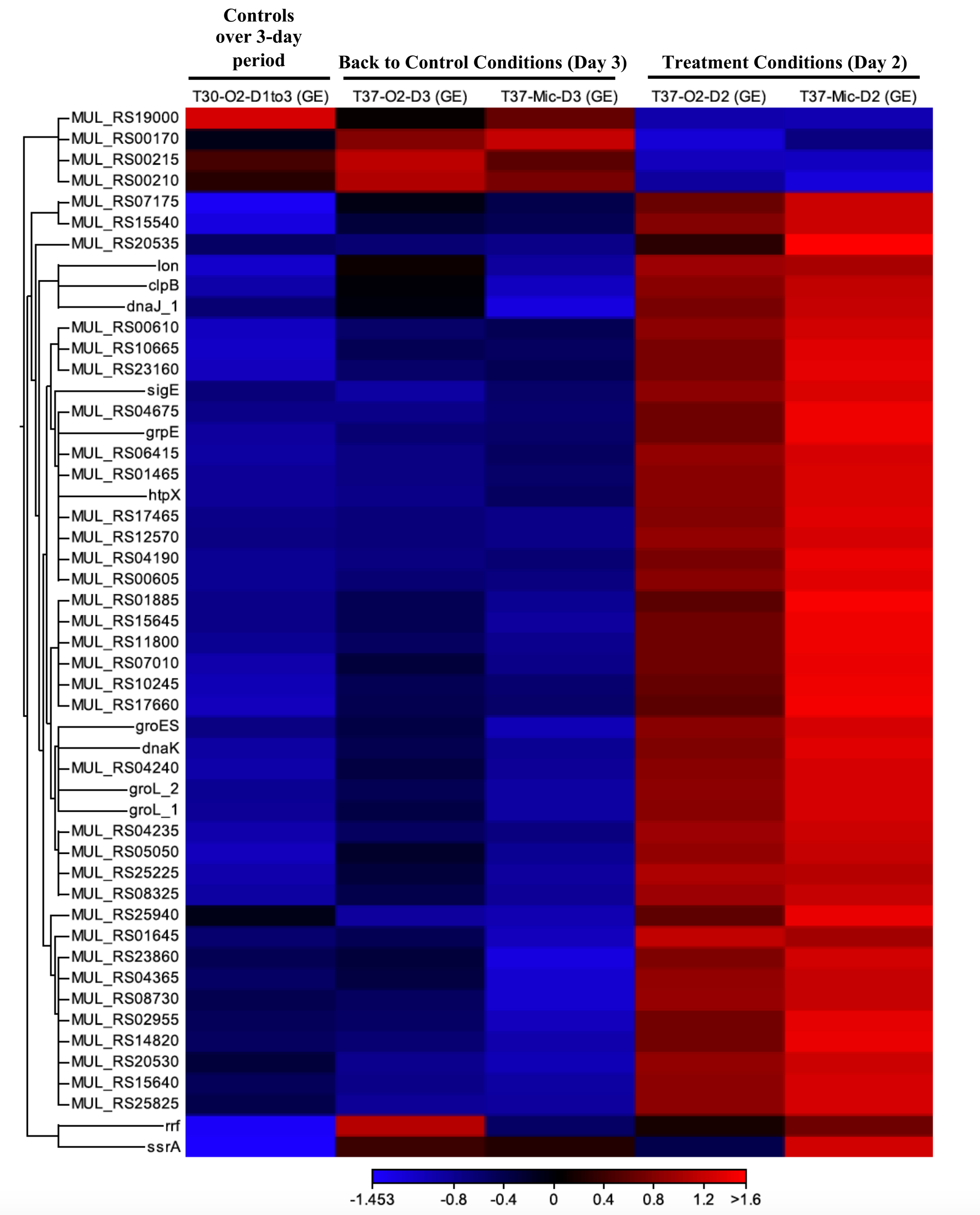

Figure 5. Fifty significantly differentially regulated genes across treatments and timepoints. T30-O2-D1-3: *M. ulcerans* grown aerobically at 30ºC for the entire 3 day study; T37-O2-D3: *M. ulcerans* grown aerobically at 37ºC for 24 hours but then moved back to control conditions for 24 hours; T37-Mic-D3: *M. ulcerans* grown microaerophilicly at 37ºC for 24 hours but then moved back to control conditions for 24 hours; T37-02-D2: *M. ulcerans* grown aerobically at 37ºC for 24 hours; T37-Mic-D2: *M. ulcerans* grown microaerophilicly at 37ºC for 24 hours.

RNASeq data showed 187 differentially regulated genes when *M. ulcerans* was grown aerobically at 37°C compared to those that remained in control conditions for the duration of the experiment. There were 158 upregulated genes including one gene involved in Environmental Informational Processing, 19 with Genetic Information Processing, 45 with Metabolism, and 4 with Signaling and Cellular Processes. Two genes were uncharacterized, and 87 had no KEGG orthology (KO) assigned. Twenty-nine genes were significantly downregulated compared to *M. ulcerans* in control conditions, including 3 involved in Environmental Informational Processing, 4 with Genetic Information Processing, 13 involved in Metabolism (3 of which were MUP001 plasmid genes MUP032c, MUP039c, and MUP040c, encoding for MLSB, MLSA2 and MLSA1, respectively), and 2 involved in Signaling and Cellular Processes (4, 24). Seven significantly downregulated genes had no KO assigned (Figure 5, Supplemental Figure 1 and Supplemental Table 1).

M. ulcerans moved back to control conditions on day 3 from being grown aerobically at 37°C showed only 12 significantly upregulated genes compared to *M. ulcerans* grown under control conditions for the entire experiment. These included 5 genes involved in Genetic Information Processing, 6 genes involved in Metabolism (including MUP032c and MUP039c), and 1 with no KO assigned. Only seven genes were significantly downregulated compared to those under control conditions including 4 involved in Metabolism, and 3 with no KO assigned (Figure 5, Supplemental Figure 1 and Supplemental Table 1).

When *M. ulcerans* was grown microaerophilicly at 37°C there were 372 significantly and differentially regulated genes compared to *M. ulcerans* grown in control conditions for the entire experiment. A total of 349 genes were upregulated including 3 genes involved in

Environmental Informational Processing, 38 involved in Genetic Information Processing (including MUP001 plasmid gene MUP005c, encoding a possible chromosome partitioning protein ParA) (27), 110 with Metabolism, and 7 with Signaling and Cellular Processes. Three genes were uncharacterized, and 188 had no KO assigned. Twenty-three genes were significantly downregulated compared to control conditions including 2 involved in Environmental Informational Processing, 2 with Genetic Information Processing, 11 involved in Metabolism (3 of which were MUP001 plasmid genes MUP039c, and MUP040c, encoding for MLSA2 and MLSA1, respectively), and 1 involved in Signaling and Cellular Processes. Seven significantly downregulated genes had no KO assigned.

When *M. ulcerans* was returned to control conditions on day 3 after being grown microaerophilicly at 37°C there were only 2 significantly upregulated genes compared to *M. ulcerans* grown under control conditions for the entire experiment. These upregulated genes included 2 gene involved in Metabolism, and 1 with no KO assigned. Only 5 genes were significantly downregulated compared to control, with all 5 being involved in Metabolism, including MUP032c.

Finally, comparison of gene expression among *M. ulcerans* growing at 37°C either aerobically or microaerophically showed 76 genes that were upregulated under the microaerophilic conditions compared to aerobic condition. These included 12 genes involved in Genetic Information Processing, 28 genes involved in Metabolism, 4 involved with Signaling and Cellular Processes, and 32 with no KO assigned. There were no statistically significant differences in downregulated *M. ulcerans* genes between either of the oxygen treatment conditions grown at 37°C.

Discussion

The capacity of *Mycobacterium ulcerans* to sense, respond and adapt to variable and hostile environmental conditions inevitably makes it successful in its natural environment and increases its ability to survive in its host. With this in mind, we conducted one of the first studies investigating *M. ulcerans* growth and transcriptional response (including modulation of mycolactone gene expression and global responses) to the abiotic conditions of UV, temperature, and oxygen, in an effort to better understand the *M. ulcerans* environmental niche and factors promoting *M. ulcerans* pathogenesis.

Premature termination of the *crt*I gene in *M. ulcerans* that protects its progenitor, *M. marinum,* against sunlight damage suggests *M. ulcerans* either resides in UV protected areas or there is presence of other machineries to counteract the damage (28, 29). However, in our study, *M. ulcerans* growth was not affected by UV radiation exposure for 10 min. Further investigations exposing *M. ulcerans* to longer duration is needed to confirm its resistance to UV radiation over longer exposure times. Although our study showed downregulation of mycolactone (ER) expression on UV exposure via RT-qPCR, this was not significant. Under laboratory conditions, wild-type *M. ulcerans* produces bright yellow pigmented colonies, while mycolactone mutants are white, suggesting mycolactone mediated protection may be possible (4, 25). In our study, the color of UV treated *M. ulcerans* colonies were yellow on M7H10 agar plates (data not shown) indicating mycolactone production. Quantitation of mycolactone production and the use of a mycolactone negative mutant will further elucidate the effect of UV beyond gene expression. Further studies on transcriptional analysis are also required to understand regulation of other machineries that can confer photoprotection and/or DNA repair to protect *M. ulcerans* against UV damage.

Exposure to higher temperature and lower than optimal oxygen conditions is also known to modulate stress response and virulence genes in environmental pathogens that can aid in the bacterial survival and pathogenicity inside the human host (30, 31). Differences in oxygen conditions did not impact *M. ulcerans* growth in our study. *M. ulcerans* exposure to anaerobic conditions caused slight upregulation of ER gene expression, however, was statistically non-significant. But when *M. ulcerans* was brought back to aerobic conditions 24 hours later, ER gene expression was significantly upregulated. The combining of anaerobic and 37^0C conditions caused significant downregulation of the ER gene that was significantly upregulated when brought back to aerobic and 30^0C conditions. *M. ulcerans* lacks anaerobic pathway genes and because of this are suggested to be adapted to an aerobic or microaerophilic environmental niche (28). However, its association with mammalian feces and asymptomatic gut colonization raises questions regarding viability, yet lack of pathogenicity in the anaerobic intestinal environment (32-37). Although the single stress anaerobic condition did not affect mycolactone gene expression, combining high temperature and anaerobic conditions downregulated mycolactone gene expression in our study, and may account for the lack of pathogenesis in the mammalian intestine. Indeed, in *S. aureus*, another pathogen system, toxic shock syndrome toxin 1 (TSST-1) was produced in a CO_2 environment (38, 39), increasing production when there was a shift from anaerobic to increasing oxygen concentrations (2%); however, toxin production was decreased for increasing oxygen concentration (6%) in an environment without CO_2 (39). Similarly, slight reduction in oxygen concentrations in microaerophilic environments could enhance ER gene expression but this effect may not occur at 0% oxygen in anaerobic environments, suggesting the possibility of a very narrow niche of *M. ulcerans* and mycolactone expression.

Under the 30^0C-microaerophilic condition, the ER gene was significantly upregulated, but was downregulated when brought back to aerobic conditions. On the other hand, ER was significantly downregulated when *M. ulcerans* was placed at 37^0C-aerobic then significantly upregulated when brought back to 30^0C-aerobic. Further, our *M. ulcerans* RNASeq data from the 37^0C-aerobic treatment showed downregulation of *mlsA1*, *mlsA2*, and *mlsB*, type I polyketide synthase genes whose proteins synthesize the upper side chain and macolactone core, and the acyl side chain, respectively (4, 24). Exposing *M. ulcerans* to combined microaerophilic and 37^0C stress conditions led to ER gene downregulation that was not statistically significant through RT-qPCR, though *M. ulcerans* RNASeq data under these combined conditions showed significant downregulation of *mlsA1* and *mlsA2*. These data contrast with our initial hypothesis that predicted upregulation of mycolactone in response to combined effects of high temperature and low oxygen conditions which we presumed to be similar in the subdermal environment during human infection.

When considering how our *in vitro* data could fit within the infection model, one must consider the skin environment. The partial pressure of oxygen (PO_2) is lower at different layers of skin (superficial-8.0 ± 3.2 mmHg, dermal papillae- 24.0 ± 6.4 and sub-papillary plexus-35.2 ± 8.0) compared to PO_2 at atmosphere (160 mmHg) (40). The sudden exposure to higher temperature and lower oxygen conditions depending on the skin layer could induce adaptive stress response mechanisms in *M. ulcerans* causing it to use its energy efficiently for production of enzymes and other proteins, and lipids. Triggering appropriate responses that allow survival and propagation under these conditions could compromise mycolactone synthesis, while also directing the expression of other genes modulating virulence and pathogenicity. However, these are speculations that require much deeper examination for mechanistic validation.

Nevertheless, the combined stress of 37^0C-microaerophilic conditions upregulated genes for lipid metabolism (Acyl-CoA dehydrogenases) and mycolic acid synthesis (*acc*D6, *mas*, *eth*A), similar to what has been shown in *M. bovis* and *M. tuberculosis* (41-43). Another upregulated gene, *eth*, encodes oxidoreductase that oxidizes keto-mycolic acid to synthesize waxy mycolic acids (44). The upregulation of these genes in our study suggests that combined temperature-oxygen stress caused disturbance in overall *M. ulcerans* metabolism and cell wall synthesis. Upregulation of the *mms*A gene, which generates propionyl CoA that produces fatty acids required for cell envelope formation suggests effects of combined stress on the cell membrane (45-47).

Genes encoding transcriptional regulators SigB, SigE and WhiB5 were also among significantly upregulated genes in *M. ulcerans* compared to controls within the 37^0C-microaerophillic treatment. Sigma B is one of the principal sigma factors and is considered a general stress responder in mycobacteria (48, 49). SigB also positively regulates expression of chaperonins such as *gro*EL2 and *gro*ES, antigens such as ESAT-6-like proteins and cell-wall associated and lipid metabolism related genes in *M. tuberculosis* (50). WhiB proteins are redox sensing transcriptional regulators (51). WhiB5 positively regulates 58 genes including type VII secretion systems (ESX-2 and 4) (51). WhiB5 proteins in *M. tuberculosis* are relatively stable and under-expressed in aerobic conditions but are slightly upregulated at 0% oxygen (52). They are suggested to be immunomodulators and enhancers of *M. tuberculosis* survival during nutrient limitation (51).

Finally, stress response genes *hsp*20, *grpE, clp*B, *gro*ES, *groEL1* and *groEL2*, dnaJ, *dna*K *ahpC,* and *ahp*D were also among those upregulated on exposure of *M. ulcerans* under combined 37^0C -microaerophilic conditions. These genes respond to heat shock and oxidative stress (41, 49, 53). The *clp*B gene is associated with virulence in several gram positive (e.g. *S. aureus*) and negative pathogens (e.g. *Salmonella enterica* serovar typhimurium) (54). In *M. tuberculosis*, *clp*B enhances biofilm formation and promotes survival against hypoxia, heat stress and inside macrophages (54). Similarly, *dna*K provides *M. tuberculosis* protection against heat shock and oxidative stress inside the macrophage (43, 55). Proteins ClpB, GroES, GroEL1, DnaK, AhpC, and AhpD are found in the extracellular matrix (ECM) of *M. ulcerans* biofilm, and *M. ulcerans* with ECM have higher colonization and virulence (56). Upregulation of these *M. ulcerans* genes *in vitro* suggests that environmental signals such as temperature, oxygen, UV, pH, etc. could trigger adaptive responses of *M. ulcerans* to these stresses during infection, or within polymicrobial communities in its natural environment. These genes could promote biofilm formation and enhance colonization and virulence activity of *M. ulcerans.* And, although mycolactone is considered as a major virulence factor, more investigations are needed to understand the impact of genes (and their products) such as *clp*B and others that are known virulence determinants in other bacteria. Another important consideration is that in this study we defined higher temperature and lower than optimal oxygen conditions as “stressed conditions” for *M. ulcerans* based on current knowledge about its growth in lab conditions; however, these environmental conditions may not be a “stressed environment” for *M. ulcerans* in its natural habitat and *M. ulcerans’s* response to fluctuating temperature and oxygen conditions may simply be a “response” instead of “stress response”.

Many of the same genes discussed above that were upregulated in the 37^0C-microaerophilic treatment were also upregulated in the 37^0C-aerobic treatment. But interestingly, no statistical differences in downregulated genes were found when comparing combined stress 37^0C-microaerophilic to single stress of 37^0C-aerobic treatment. Altogether, our data suggest a higher impact of temperature than oxygen on *M. ulcerans* and mycolactone expression. One possible explanation may be that the microaerophilic conditions induce upregulation of mycolactone expression, but the combined stress of high temperature and low oxygen prioritizes the expression of other essential genes (i.e. stress response and fatty acid degradation genes as discussed above), thereby compromising mycolactone synthesis. This is a mechanism found in other pathogens. For instance, in *Vibrio cholerae*, the promoter of cholera toxin regulator (*tox*R) and heat shock (*htp*G) genes overlap but are transcribed in opposite directions (57, 58). Hence, increase in temperature allows transcription of the *htp*G gene with reduction in *tox*R expression. Another consideration is, in *M. ulcerans*, the ER domain is present repeatedly in the *mls*A gene (3 times in *mls*A1 and 1 time in *mls*A2); however, this domain is absent in the *mls*B gene (4). Hence, ER represents one of many possible genes involved in mycolactone synthesis, but its regulation does not solely depict mycolactone gene expression. Thus, this may account for the discrepancy in RT-qPCR and RNASeq results for mycolactone expression.

Finally, data support that mycolactone producing mycobacteria (MPM) such as *M. ulcerans* and other ecological variants evolved from a common *M. marinum* progenitor by undergoing various gene deletions, pseudogene formation and rearrangement, and acquiring plasmid pMUM to adapt to a specific ecological niche (59). In the evolutionary hierarchy, *M. liflandii* is suggested to be an intermediate between the ancestor *M. marinum* M and *M. ulcerans Agy*99, as *M. marinum* consists of all gene cluster present in *M. liflandii* and *M. liflandii* consists of all gene cluster present in *M. ulcerans* (60). But there are some genes that are pseudogenized in *M. ulcerans* but not in *M. liflandii* and vice-versa, indicating that there was a significant and independent reductive evolution of their genomes. These differences in mutation patterns along with the variation in type of mycolactone produced indicates that these variants experience different sets of environmental pressure and have adapted to occupy different niches, underscored by differing hosts (6, 61). Therefore, it would be interesting to determine whether these responses are also observed for other MPMs.

In conclusion, *M. ulcerans* acquired plasmid pMUM001 at the expense of a large deletion in its genome thereby suggesting its specific role in adaptation to a particular environment (59). In this study, *M. ulcerans* was exposed to several abiotic stresses to understand their effect on *M. ulcerans* growth, and mycolactone and other gene expression. Our data suggest that *M. ulcerans* may reside in a microaerophilic habitat in the environment and mycolactone could provide a fitness advantage in those environments. Data also suggest that *M. ulcerans* may have higher tolerance to UV than previously thought and mycolactone may provide a fitness advantage in this context. The exposure of *M. ulcerans* to combined high temperature (37^0C) and low oxygen upregulated several stress response genes and other genes known to be involved in virulence in *M tuberculosis,* while mycolactone gene expression was downregulated-suggesting other virulence factors may be utilized by *M. ulcerans*. Further, downregulation of mycolactone expression on exposure to combined anaerobic and 37^0C could partly explain the reason behind the asymptomatic gut colonization of different mammals as reported in previous studies (35). Some limitations to this study such as small sample size, short duration exposure of *M. ulcerans* to UV radiations (up to 10 mins) and no transcriptome analysis to study other machineries that can protect *M. ulcerans* against UV damage should be addressed in future studies. Additionally, short time points were selected for this study to understand the impacts of short term exposure on *M. ulcerans* response through changes in gene expression; however, measuring responses to longer exposure times will be important future experiments. Finally, investigations measuring mycolactone production, and using a mycolactone negative *M. ulcerans* mutant and other MPMs are needed to further elucidate the role of mycolactone against these and other abiotic stresses. But altogether, these initial data increase our understanding of *M. ulcerans* response to a changing environment and open doors to future studies that may provide insight to the *M. ulcerans* environment and pathogenesis upon host infection.

Materials and methods

Bacterial strains and culture

A 1% inoculum of *Mycobacterium ulcerans* JKD8083 or *Agy*99 was inoculated into 30mL total volume Middlebrook 7H9 (M7H9) broth containing Oleic Albumin Dextrose Catalase (OADC) and incubated aerobically at 30^0C for 4-6 weeks to reach exponential phase for use in this study.

Measurement of optical density (OD600)

M. ulcerans cells form aggregates in culture. Hence, aggregates were broken by passage through a 20G syringe, followed by a 25G syringe 10 times. Optical density was measured using a ThermoScientific Genesys 20 spectrophotometer, with M7H9 medium used as a blank. Syringe passage of *M. ulcerans* was used for all experiments.

Measurement of bacterial growth

M. ulcerans was serially diluted in $\log_{10}$ concentrations (from undiluted to 10^{-6}) and plated onto M7H10 agar plates (10 uL) in triplicate using the spread plate technique and incubated at 30°C. Colonies were counted to determine CFUs/mL after 4-6 weeks to determine the effect of the abiotic factors on *M. ulcerans* growth, and a mean was taken for the triplicates, with standard errors calculated.

Effect of UV exposure on *M. ulcerans* growth and mycolactone gene expression

Mycolactone expression was measured on exposure to UV at different time intervals (30s, 2 min and 10 min). The time for UV exposure was determined based on work by David *et al.* (1971), where *M. tuberculosis* and *M. marinum* were exposed to UV for up to 30 seconds and were inactivated (90%) by 7 seconds and 22 seconds UV exposure, respectively (62). *M. ulcerans* during exponential growth (5.7 x 10^5 CFU/mL, 30 mL) was transferred to individual Petri plates and exposed to UV (254 nm wavelength) for their respective time interval to measure *M. ulcerans* growth and modulation of mycolactone gene expression. Controls included *M. ulcerans* transferred to Petri plates, but without UV exposure. At each timepoint and condition, 1 mL of *M. ulcerans* was transferred for serial dilution and growth measurement by optical density and CFU count, and 5.0 mL of sample was transferred for RNA isolation and RT-qPCR to measure mycolactone gene expression as described below.

Effect of temperature and oxygen on *M. ulcerans* growth and mycolactone gene expression

To determine the effects of different oxygen conditions on *M. ulcerans* growth, *M. ulcerans* initially grown aerobically at 30°C were exposed to either microaerophilic or anaerobic conditions at 30°C for 24 hours, by placing those cultures within anaerobic chambers with appropriate gas paks (BD) and oxygen indicators (Figure 6A). Optical density was measured initially, and after 24 hours. *M. ulcerans* cultures were then placed back in aerobic conditions at 30°C for an additional 24 hours (48 hours from initial time point). The optical density for each treatment and timepoint was measured. To study the effects of temperature, *M. ulcerans* in exponential growth aerobically at 30^0C were exposed to 37°C for 24 hours and then placed back in the 30°C incubator for an additional 24 hours. The optical density was measured for each treatment and time point. At each timepoint and condition, 1 mL of sample was transferred for serial dilution and growth measurement, and 5.0 mL of sample was transferred for RNA isolation and RT-qPCR to measure mycolactone gene expression. Each experiment was conducted with triplicate replicates and at least three times.

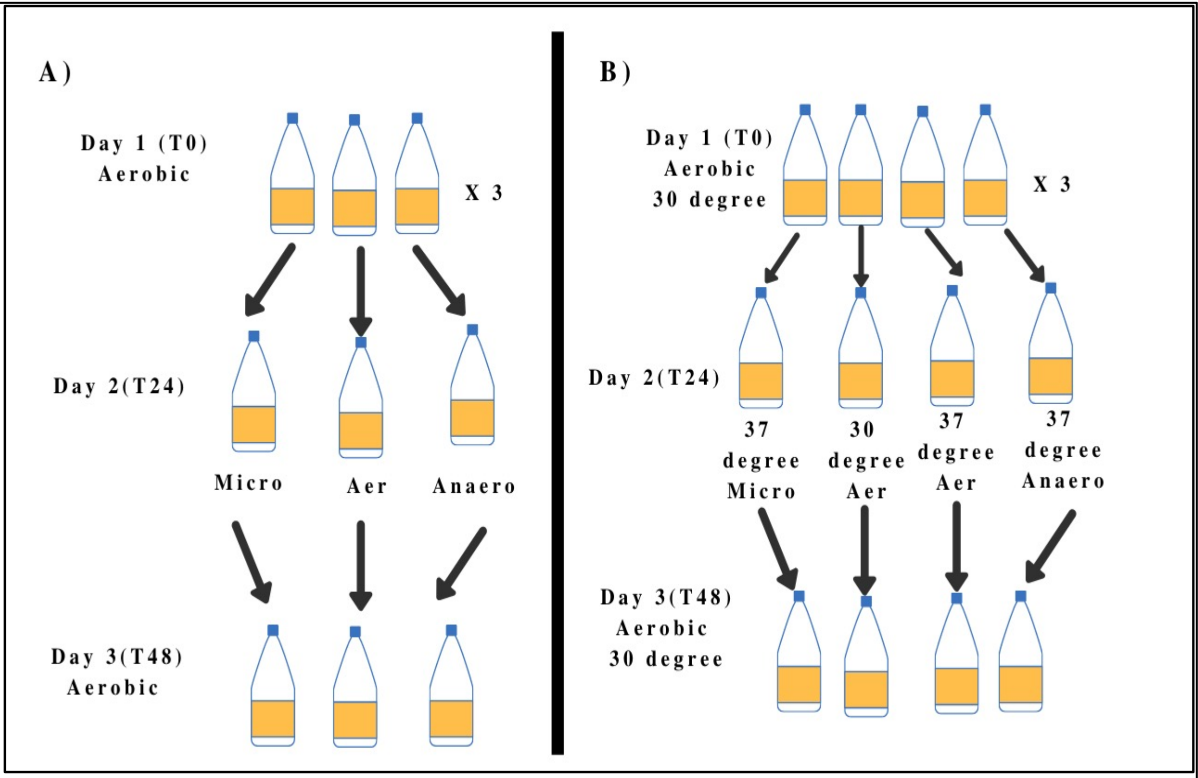

Figure 6. Schematic representation of study on the effect of oxygen (A) and combined high temperature (37^0C) and low oxygen (microaerophilic and anaerobic) condition (B) on mycolactone and *M. ulcerans* global gene expression.

Combined effect of high temperature and low oxygen in mycolactone and global gene expression

Exponentially grown *M. ulcerans* (4-6 weeks) aerobically and at 30°C were exposed to 37^0C under microaerophilic or anaerobic conditions for 24 hours (Figure 6B). After 24 hours, the cultures were again brought back to control conditions (30°C and aerobic) for an additional 24 hours (48 hours from initial timepoint). Optical densities were measured for each condition and timepoint, with samples also collected and analyzed as described above. Each experiment was conducted with triplicate replicates (Figure 6B).

RNA isolation

M. ulcerans RNA was isolated using the Trizol method, according to manufacturer instructions. Bacterial cells were pelleted by centrifugation, with supernatant removed, and 1.0 mL Trizol reagent was added to the pellet and mixed thoroughly and bead beaded. After incubation for one hour, chloroform was added and centrifuged for phase separation. The aqueous phase containing RNA was obtained and precipitated using isopropanol followed by washing with 75% ethanol. The pellet was dried and dissolved in nuclease-free water to obtain RNA suspension. RNA was quantified using the qubit 2.0, integrity verified by gel electrophoresis, and DNase treated as necessary.

Preparation of cDNA

cDNA was prepared with appropriate controls using the Verso cDNA Synthesis kit according to the manufacturer's instructions. The reaction mixture included 4 µl synthesis buffer, 2 µl dNTP mix, 1 µl random hexamer, 1 µl verso enzyme and 1 µl RT enhancer (to prevent genomic DNA carryover) was added to the template and heated at 42^0C for one hour to obtain cDNA. Absence of genomic DNA was confirmed by qPCR.

Quantitative Real Time PCR (RT-qPCR)

RT-qPCR targeting the enoyl reductase (ER) domain of module B of pMUM001 responsible for mycolactone production was performed on cDNA (15, 63, 64). The polyphosphate kinase (*ppk*) gene was used as a reference gene (25). The master mix contained one µl of each forward and reverse primer for *ppk* gene and ER gene, 2.5 µl of ER probe and *ppk* probe, 12.5 µl of master mix and 0.5 µl water and 3 µl template cDNA per well of PCR plate. The forward primer for ER was 5’CGCCTACATCGCTTTGG3’ and reverse primer 5’ATTGAATCGCAGCCATACC3’. The forward *ppk* primer was 5’ CGGGAAACTACAACAGCAAGACC 3’ and the *ppk* reverse was 5’ CCACCAACAGATTGCGATAGG 3’. PCR was conducted on triplicate samples using a BioRad CFX96 with parameters that include 95.0°C for 10:00 min, and 39 cycles of 95.0°C for 15 seconds, 55.0°C for 30 seconds, 57.0°C for 30 seconds.

RNASeq Analysis

RNA libraries were created from combined triplicate replicates of *M. ulcerans* RNA samples under aerobic and 30^{0}C (T0-T48H), aerobic and 37^{0}C (T24 H), microaerophilic and 37^{0}C (T24 H), and samples that were transferred from 37^{0}C, aerobic or microaerophilic conditions to aerobic and 30^{0}C at 48 H [aerobic and 37^{0}C (T48 H), microaerophilic and 37^{0}C (T48 H)] conditions. Libraries were created using the NEBNext® Ultra™ RNA Library Prep Kit and NEBNext® Multiplex Oligos (Dual Index Primers) for Illumina® and associated protocols. High-throughput RNA sequencing was performed by St. Jude Children’s Research Hospital on an Illumina HiSeq2000 with 2 X 150bp PE (paired end) read lengths. Sequences were initially trimmed by the sequencing facility using TrimGalore v0.4.2 but a more stringent quality trimming was also performed using default parameters within the Qiagen CLC Workbench 20.0.1 (https://www.qiagenbioinformatics.com/) following QC analysis of sequence reads. *M. ulcerans Agy*99 and plasmid pMUM001 reference genomes were joined (References Sequence NC_008611 joined with Reference Sequence NC_005916, Assembly GCF_000013925.1, https://www.ncbi.nlm.nih.gov/assembly/GCF_000013925.1), and RNASeq data were mapped with the following parameters: (a) maximum number of allowed mismatches was set at 2, with insertions and deletions set at 3; (b) Length and similarity fractions were set to 0.9, with autodetection for both strands; (c) minimum number of hits per read was set to 10. All genes listed in heatmaps are labeled as listed in RefSeq for a particular locus tag given in the reference genomes' annotation.

Differential expression was measured in the CLC Workbench that used the assumption that transcripts with similar average expression levels had similar variability, according to the CLC Manual. Statistical differential expression tests were performed based on a negative binominal generalized linear model similar to that of edgeR (65). Differentially expressed genes were generated based on a false discovery rate -corrected *p*-value (FDR) using the Wald test for comparing the effects of treatments compared to control, or treatments compared across time. Treatment reads with a fold change of 1.5 or higher, and FDR adjusted P-value less than or equal to 0.05 were considered significant (66). Statistically significant, differentially regulated gene transcripts were further annotated into pathways by linking protein ID with potential conserved domains and protein classifications archived within the Conserved Domain Database ("Conserved Domains and Protein Classification," n.d.), and by using the UniProt ("UniProt," n.d.), KEGG (Kanehisa Laboratories, n.d.), and STRING databases (ELIXIR Core Data Resources, n.d.), gene annotations within NCBI (https://www.ncbi.nlm.nih.gov/nuccore/NC_008611.1 and https://www.ncbi.nlm.nih.gov/nuccore/NC_005916), mycolactone locus patent information (https://www.freepatentsonline.com/y2006/0024806.html) and the Mycobrowser genomic and proteomic database for reference against other mycobacterial species (https://mycobrowser.epfl.ch/genes/Rv0753c).

Statistical analysis

Significant difference changes in *M. ulcerans* growth under control conditions compared to abiotic treatment conditions were determined using a student's T-test. RT-qPCR data were analyzed by relative quantification of gene expression compared to the control using python code implementing the ΔΔCT method (67). The reference gene used was *ppk* and ER was used for the target gene. The fold change in gene expression was determined to obtain regulation relative to control (baseline). If the fold change relative to control was greater than 1 then it was considered upregulated. If fold change was less than 1 then it was considered downregulated. The amount of downregulation (for fold change 0 to 1) was determined by calculating negative of the reciprocal of fold change, as described by Babu, 2004 (68). The significant cut-off value (α) for upregulation and downregulation was $p=0.050$.

Data Availability

Raw sequences are archived in the NCBI Sequence Read Archive (SRA) under Bioproject Accession number: PRJNA907849.

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Figure Legends

Figure 1. Effects of increasing UV exposure on *M. ulcerans* growth (A) and ER expression (B).

Figure 2. Effect of Oxygen (A) or temperature (B) on *M. ulcerans* growth. (A) Optical density of *M. ulcerans* when exposed to aerobic (blue line), microaerophilic (orange) and anaerobic (grey line) conditions at 30^0C. Exponential *M. ulcerans* initially under aerobic conditions were exposed to their respective oxygen condition for 24 hours and then transferred back to aerobic conditions for an additional 24 hours (48 hours from initial time point). (B) Optical density of *M. ulcerans* when exposed to 30^0C (blue line) and 37^0C (orange line). Exponential *M. ulcerans* initially at 30^0C were exposed to their respective temperature conditions for 24 hours and then exposed back to 30^0C for an additional 24 hours (48 hours from initial time point).

Figure 3. *M. ulcerans* ER regulation after exposure to microaerophilic or anaerobic conditions compared to aerobic conditions. Exposure to a microaerophilic environment for 24 hours caused significant upregulation [Mic (24H); P=0.0009] of ER expression (yellow bar) and transferring the bacteria back to aerobic condition led to slight downregulation in ER expression [Mic (48H), grey bar]. Exposure of *M. ulcerans* to anaerobic conditions for 24 hours led to slight ER gene upregulation [AN (24H), blue bar], but transfer back to aerobic conditions caused significant ER upregulation [AN (48H), P=0.005, red bar] compared to control *M. ulcerans* exposed to aerobic conditions during the entire 3-day experiment (Control bar). Error bars indicate Standard errors. *M. ulcerans* was at 30º C for all the oxygen conditions.

Figure 4. Regulation of ER expression after *M. ulcerans* exposure to differing temperature and oxygen conditions. (A) Control conditions of *M. ulcerans* at 30^0C and aerobic conditions from days 1-3; (B and in blue box): *M. ulcerans* exposed to 37^0C and aerobic conditions on Day 2 [T37-O2 (D2)] but transferred back to 30^0C and aerobic condition on day 3 [T30-O2 (D3)]; (C and in red box): *M. ulcerans* exposed to 37^0C and microaerophilic condition on day 2 [T37-Mic (D2)] but transferred back to 30^0C and aerobic condition on day 3 [T30-O2 (D3)]; (D and in green box): *M. ulcerans* exposed to 37ºC and anaerobic condition on day 2 [T37-Anae (D2)] but transferred back to 30ºC and aerobic condition on day 3 [T37-O2 (D3)]: Bars indicate standard error. P values indicate significance values between treatment and control. Stars indicate significance within treatments.

Figure 5. Fifty significantly differentially regulated genes across treatments and timepoints. T30-O2-D1-3: *M. ulcerans* grown aerobically at 30ºC for the entire 3 day study; T37-O2-D3: *M. ulcerans* grown aerobically at 37ºC for 24 hours but then moved back to control conditions for 24 hours; T37-Mic-D3: *M. ulcerans* grown microaerophilicly at 37ºC for

24 hours but then moved back to control conditions for 24 hours; T37-02-D2: *M. ulcerans* grown aerobically at 37ºC for 24 hours; T37-Mic-D2: *M. ulcerans* grown microaerophilicly at 37ºC for 24 hours. All genes listed in the heatmap are labeled as listed in RefSeq for a particular gene or locus tag given in the reference genomes' annotation.

Figure 6. Schematic representation of study on the effect of oxygen (A) and combined high temperature (37^{0}C) and low oxygen (microaerophilic and anaerobic) condition (B) on mycolactone and *M. ulcerans* global gene expression.