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Investigations on mechanisms of survival and pathogenesis of Mycobacterium ulcerans in polymicrobial environments

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Investigations on mechanisms of survival and pathogenesis of *Mycobacterium ulcerans* in polymicrobial environments

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Buruli ulcer disease (BUD) remains a 'mysterious disease' due to the unknown mode of *M. ulcerans* transmission and pathogenesis. To understand these, it is important to determine the reservoir of the organism in its natural environments, and stress response and interactions of *M. ulcerans* in its natural niche and during infection of a host. The major virulence factor of *M. ulcerans* is mycolactone, a lipid cytotoxin that is encoded on a giant plasmid pMUM001. Genetic analysis suggests that plasmid pMUM001 was acquired by *M. ulcerans* during evolution from its progenitor, *M. marinum*. Coincidental evolution of virulence hypothesis suggests that many microbes evolve to acquire traits to outcompete or overcome biotic and abiotic forces during their normal life cycle in the outside-host environment, which can confer virulence during infection of a human host. Hence in this study, we exposed *M. ulcerans* to selective abiotic forces such as UV, and dynamic oxygen and temperature conditions to determine their effect on *M. ulcerans* growth, and mycolactone and global gene expression. We also studied the role of mycolactone in determining polymicrobial interaction of *M. ulcerans* in its natural aquatic habitat by exposing mycolactone coated and uncoated slides in *M. ulcerans* endemic and non-endemic aquatic locations and determining differences in microbial community composition between them.

Further, we studied quorum quenching ability of mycolactone against an opportunistic pathogen, *S. aureus.* The results obtained showed that exposure of *M. ulcerans* to abiotic stresses such as higher temperature and lower than optimal oxygen conditions modulate its global and mycolactone gene expression. Further, we also showed that mycolactone can impact overall microbial community structure in a polymicrobial environment in its natural, aquatic habitat. Mycolactone also effected virulence and quorum sensing in an opportunistic pathogen, *S. aureus,* without inhibiting its growth. These findings are important as they provide insight toward potential reservoirs or environmental niches which may harbor *M. ulcerans* and inform new potential mechanisms of pathogenesis. Further, our novel research of synergistic or antagonistic interactions within the complex polymicrobial communities colonizing skin and aquatic habitats is a powerful approach in determining *M. ulcerans* colonization efficiency, resiliency, and transmission mechanisms.

DEDICATION

I would like to dedicate this dissertation to my loving husband Dr. Durga Prasad Siwakoti and son Dibesh Siwakoti who have been and remain my guidance, support, and inspiration. To be loved by you makes me feel one of the luckiest in the world.

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CHAPTER I

LINKING THE MYCOBACTERIUM ULCERANS ENVIRONMENT TO BURULI ULCER DISEASE: PROGRESS AND CHALLENGES

(INTRODUCTION)

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1.1 Abstract

Buruli ulcer disease (BUD), the second most common mycobacterial disease in West Africa, is a necrotizing skin disease that can lead to high morbidity in affected patients. The disease is caused by *Mycobacterium ulcerans,* whose major virulence factor is mycolactone. Although infection can be treated with antibiotics, an effective preventative strategy is challenging due to unknown reservoir(s) and unresolved mode of transmission. Further, disease occurrence in remote locations with limited access to health facilities further complicates disease burden and associated costs. We discuss here severity and socio-economic impact of BUD along with *M. ulcerans* microbiological research. We also highlight *M. ulcerans* transmission hypotheses and investigations into environmental reservoirs and discuss successes and challenges of studying *M. ulcerans* and Buruli ulcer across human, animal, and environmental interfaces. We argue that a One Health approach is needed for advances in resolving the mode of transmission and designing management scenarios that prevent and respond to epidemics.

Although previous work has provided significant insights on risk factors, epidemiology and clinical perspectives of disease, understanding the bacterial ecology, environmental niches and role of mycolactone in natural environments and during infection of the human host remains equally important to better understanding and preventing this mysterious disease.

1.2 Introduction

Buruli ulcer disease (BUD), one of the 17 neglected tropical diseases (NTDs) recognized by the WHO, is caused by an environmental pathogen *Mycobacterium ulcerans.* BUD is a necrotizing skin disease that initially presents as a painless nodule or papule, that can develop into ulceration in later stages (Williamson et al., 2012, 2014). The ulcerative stage is characterized as an extensive necrotic ulcer with undermined edges. Pathogenesis is caused by mycolactone, a cytotoxic lipid compound with genes found on a plasmid. The disease mainly occurs at the dermal and subdermal layers of the skin, particularly on upper and lower extremities, however, osteomyelitis affecting distant metastases has also been reported (Lagarrigue, Portaels, Meyers, & Aguiar, 2000; World Health Organization, 2018a). It is the third most common mycobacterial infection after tuberculosis and leprosy and second most common mycobacterial disease in West Africa (Koka, Yeboah-Manu, Okyere, Adongo, & Ahorlu, 2016). In West Africa, the disease is prevalent in children under age 15 and serious morbidity such as functional disabilities and disfigurement is reported (Maman et al., 2018). In Australia, the epidemiology is different, with the majority of patients older than 49 years of age. Before 2004, the disease was mainly treated by surgical excision of affected tissue, however, introduction of streptomycin and rifampicin in combination as an antibiotic regimen showed promise to treat BUD at its initial stage and prevention of progression to the ulcerative stage (Adu, Ampadu, & Acheampong, 2011). But, Streptomycin is administered intramuscularly and

can cause nephrotoxicity and toxicity, so other treatment regimes have been sought (Klis et al., 2014; Phillips et al., 2020). Currently, the WHO recommendation for treatment of BUD includes a combination of rifampicin and clarithromycin (World Health Organization, 2020b). A non-inferiority phase 3 trial conducted by Philips *et al*. (2020) in patients older than 5 years and with ulcers no larger than 10 cm have shown that combination treatment of rifampicin and clarithromycin have less adverse effects compared to combination treatment of rifampicin and streptomycin (Phillips et al., 2020). The major hurdle to disease management is that transmission to the human host is not fully understood. Though several competing hypotheses have suggested that skin puncture or some other form of deep epidermal trauma is needed for pathogenesis, the actual method of inoculation into such openings has not been resolved. In this review we evaluate the clinical, microbiological, epidemiological, environmental and other aspects of BUD and its agent, *M. ulcerans*, drawing attention to how taking a One Health approach may lead to better disease management and prevention, and potentially leading to the discovering of the mode or modes of transmission. Developing a quantitative and multidisciplinary understanding of the role environmental and ecological changes have in disease emergence will allow for novel management scenarios related to the nexus of natural resources and human activity in endemic areas.

1.3 Epidemiology of the disease

Buruli ulcer disease has been reported in over 33 countries worldwide, however, it is primarily endemic in West and Central Africa (World Health Organization, 2019a). According to the WHO, 63,955 total BUD cases have been reported between 2002 and 2018 (World Health Organization, 2019d), with the highest number of total cases (22,193) reported from Cote d'Ivoire, followed by Ghana (12,813) and Benin (10,472). However, in 2018, the highest number

of new cases was reported in Ghana (630) followed by Nigeria (424) and Australia (358) (World Health Organization, 2019d). These data show that overall disease occurrence has decreased by about 16% from 2002 to 2018, though an exact cause of decrease in disease abundance is unknown (World Health Organization, 2020a).However, it is speculated that this may not represent the true disease burden and that poor PCR performance on confirmation of BUD may be one of the reasons for underreporting of cases (World Health Organization, 2019f). An increase in disease occurrence by about 16% was observed between 2017 and 2018. The countries showing increases in the number of BUD cases in 2018 compared to 2017 were Australia, Cameroon, Ghana, Guinea, Liberia, Nigeria, Papua New Guinea and Togo. In Australia, the Bellarine Peninsula in Victoria has had the highest BUD prevalence (Boyd et al., 2012), whereas in South America, French Guiana has reported the highest prevalence (Douine et al., 2017). The numbers of new reported cases of Buruli ulcer to WHO across geography from 2013-2018 are shown in Table 1.1.

Table 1.1 Number of new reported cases of Buruli ulcer to WHO across geography from 2013-2018

Continent/Region	**2018**	**2017**	**2016**	**2015**	**2014**	**2002-2013**
Africa	2335	2038	1748	1917	2143	51787
Central Africa	283	251	299	410	365	7936
East-Central Africa	0	0	0	0	0	1283
West Africa	2052	1787	1449	1507	1778	42568
Oceania	370	299	205	120	92	832
Australia	358	292	186	111	89	699
Papua New Guinea	12	7	19	9	3	133
Asia (Japan)	3	7	2	4	7	46
South America	No Data	No Data	No Data	No Data	No Data	No Data

The disease is mainly associated with aquatic habitats (R. W. Merritt et al., 2010). A recent study showed that disease occurred primarily in populations living near marshy savannahs, suggesting specific human contact factors within these locations with a contaminated environment (Douine et al., 2017). Specific landscape configurations have also been shown to associate with BUD prevalence (Campbell et al., 2015; Wagner, Benbow, Brenden, Qi, & Johnson, 2008; Wagner, Benbow, Burns, et al., 2008), suggesting that land use and land cover may play important roles for *M. ulcerans* abundance in the environment, perhaps through changes in the biotic and abiotic factors of associated waterbodies used by human communities (Eric Benbow et al., 2014; McIntosh et al., 2014; Pileggi et al., 2017). In Africa, BUD affects populations belonging to rural, and often impoverished groups (Roltgen, K and Pluschke, 2015), whereas in Australia the disease has been mainly associated with lifestyle and habits such as gardening, washing of wounds or visiting *M. ulcerans* contaminated lakes; and use of long trousers or insect repellant has shown to be protective (Quek et al., 2007). Furthermore, in French Guiana, certain human activities such as dam construction or rice field development have been associated with decrease or increase in BUD prevalence, respectively (Douine et al., 2017).

Infection can occur at any age-group however, it is most commonly reported in children under age 15 and adults above age 49 in Africa and Australia, respectively (O'Brien et al., 2015; Roltgen, K and Pluschke, 2015). However, a shift in higher disease prevalence from children (before 1984) to the older population (1999-2013) was recently reported in French Guiana (Douine et al., 2017). Although overall prevalence of BUD is not considered different based on sex, consideration of both age and sex in analysis has shown higher prevalence of BUD in younger males and older females (Vincent, Ardant, Adeye, et al., 2014). Also, a significant

difference in the BUD lesion site occurred with sex in French Guiana with lesions mainly located on chests and upper limbs of males and in lower limbs of females (Douine et al., 2017).

1.4 Clinical presentation of BUD

Buruli ulcer disease can present as a papule or nodule, plaque with comparatively larger size (>2cm), diffused and extensive oedematous forms, or as ulcers (Guarnera, 2018; F Portaels, Johnson, & Meyers, 2001; Yotsu et al., 2015) (Figure 1.1). The ulcerative form of BUD is characterized by undermined edges, peripheral induration and cotton-wool like appearance due to sloughing of necrotic tissue (World Health Organization, 2019b). The ulcerative form, if left untreated, can lead to disfigurement and disability (Figure 1.1). Buruli ulcer disease can also affect bone, leading to osteomyelitis, which has been shown to be painless at its initial stage but can become painful with swelling, gelatinous tissue and a "moth-eaten" appearance of bone upon incision (World Health Organization, 2019b). Despite its severity, the disease is primarily described as painless and afebrile due to the immunosuppressive property of its major virulence factor, mycolactone (Fred Stephen Sarfo, Phillips, Wansbrough-Jones, & Simmonds, 2016). However, pain and fever can occur during secondary bacterial infection and in oedematous forms (World Health Organization, 2019b). Clinical investigations of BUD show it occurs primarily on upper (35%) and lower extremities (55%) as well as in other body parts such as face and chest (10%) (World Health Organization, 2018a). A 1999 survey conducted in Ghana based on clinical presentation of BUD showed that lesions occur primarily on lower limbs followed by upper limbs, head and trunk (Amofah et al., 2002). A cohort study conducted between 2005 and 2011 in Benin showed that higher percentage of lesions occurred on lower limbs, however, younger patients (15 year or younger) had a higher chance of developing ulcers on their upper body (Vincent, Ardant, Adeye, et al., 2014). Moreover, a large-scale retrospective observational study

examining patients from Victoria, Australia showed a higher percentage of lesions located on lower limbs than upper, and showed a non-random distribution with strong predilection for the ankles, elbows and calves (Yerramilli et al., 2017).

Based on severity of disease, the WHO has classified BUD into three main categories: Category I, category II and category III (World Health Organization, 2018a). Category I includes single, small lesions (<5 cm in diameter) that can be treated by antibiotics whereas category II includes non-ulcerative and ulcerative plaque or oedematous forms or lesions with diameter in the range of 5-15 cm. Category III lesions are large lesions (>15 cm), multiple lesions, lesions at sensitive body locations such as eyes, breasts or genitalia; or that are disseminated or involving bones and joints, (WHO, 2008; World Health Organization, 2019e). Although some of the category II and category III lesions can be treated with antibiotics alone, surgery along with antibiotics may be necessary for treatment of category III lesions. And in some cases, amputation is necessary (WHO, 2008).

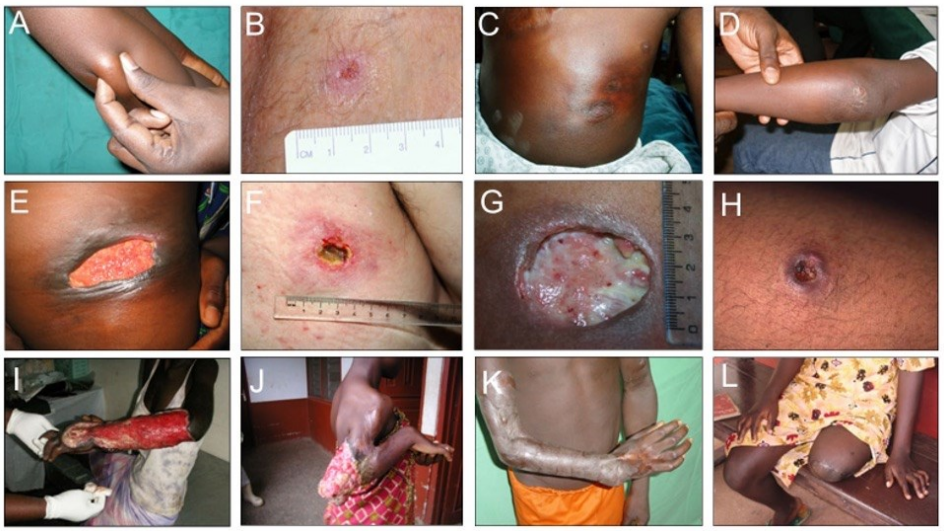

Figure 1.1 Various presentations of Buruli ulcer disease and complications.

A. Nodular form. B. Papule. C. Plaque. D. Oedema form. E-H. Small ulcers from Benin, Australia, Peru and French Guyana respectively. I. Extensive ulceration. J. Skin cancer following BUD. K. Contracture of arm following BUD. L. Amputation following BUD (that can occur in some cases). Image Source: WHO photo library (https://www.who.int/buruli/photos/en/?fbclid=IwAR2zF5IySEDbEwia5IulMPSuHk2ubXtVCmE35DE7mLZa407k3tFUKHvlkLM)

1.5 BUD in immunocompromised individuals and BUD complications

Buruli ulcer pathology affecting the face has been found to occur in frequency of 0.8-16.7%, particularly in patients of Africa (Phanzu et al., 2011). Severe complications such as facial disfigurement due to exposed teeth and gums, eye related complications such as lagophthalmos, ectropion, entropion, trichiasis and vision loss, and functional complications such as difficulties in speaking, eating and drinking, depending on the site of infection, have been reported (Phanzu et al., 2011). Stiff neck, loss of ear, nose, lower lip, eyelid, and eye ball have also been reported as a result of infection (Agbenorku, 2011). In HIV-positive-patients, multiple, large, ulcerated lesions requiring longer healing-time may develop early, especially in patients with CD4 counts <500/mm^3 (Christinet et al., 2014). Furthermore, a severe paradoxical reaction

(an adverse effect of drug therapy) in patients with HIV/BUD co-infection and being treated with antiretroviral therapy (ART)-BUD combination therapy has been reported (Smith, Hauben, & Aronson, 2012; Wanda et al., 2014). The association of BUD and HIV infection remains unclear as contradicting results of no association or increased risk of BUD in HIV patients has been shown by different studies (Christinet et al., 2014; Raghunathan et al., 2005). But, the increased severity characterized by large and multiple lesions, delayed healing time and dissemination and involvement of bone has been demonstrated in HIV-infected patients compared to non-infected patients (Christinet et al., 2014; Vincent, Ardant, Marsollier, Chauty, & Alcaïs, 2014).

Furthermore, a study in Australia by O'Brien et al. (2015) found that multiple lesions and higher numbers of BUD oedematic forms were present in elderly populations (>65 years), leading to challenges due to decreased treatment success, antibiotic related complications and paradoxical reactions (O'Brien et al., 2015). Older populations are suggested to have higher risk of BUD in Victoria, Australia (> 60 years) and in Africa (>50 years) (Avumegah et al., 2018; Bratschi et al., 2013; M. Debacker et al., 2004; Martine Debacker et al., 2006). However, in North Queensland, Australia the average age of BUD patients were 41.7 years and 50 years based on data collected from 1964- 2008 and 2013- 2018 respectively (C. Steffen, 2019; C. M. Steffen, Smith, & McBride, 2010). The occurrence of BUD in sensitive parts of immunocompromised hosts is associated with increased morbidity.

Studies have reported both co-occurrence and secondary development of squamous cell carcinoma in Buruli ulcer patients in Ghana and Cote d'Ivoire (Addison et al., 2017; Guarner et al., 2003; Kaloga et al., 2016; Kassi et al., 2010). In addition, secondary cephalic BUD with clinical presentation of painless nodules has been observed in a 4-year-old boy after antibiotic treatment (Vagamon et al., 2013). Also, secondary bacterial infection by pathogenic bacteria

such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* can co-occur with BUD, and lead to painful erythema, discoloration and delayed wound healing, particularly after administration of antibiotics (Anyim et al., 2016; Kpeli et al., 2016; Yeboah-Manu et al., 2013). Deep, painful ulcers with a budding and purulent ulcer base and inflamed and raised borders have been observed in BUD co-infected with *Leishmania braziliensis* (Mougin et al., 2011), and patients co-infected with *Mansonella perstans* (Phillips, Frimpong, et al., 2014).

1.6 Diagnosis, prevention and treatment of the disease

The diagnosis of BUD includes clinical diagnosis by health professionals, differential diagnosis to exclude other forms of ulcers such as diabetic ulcer, cutaneous leishmaniasis and yaws, and laboratory diagnosis to confirm the disease (World Health Organization, 2018a). At present, the available techniques for the laboratory diagnosis of BUD are microscopy, culture, PCR and histopathology (Eddyani et al., 2018). Each of these techniques has its own advantages and disadvantages in terms of sensitivity, cost and convenience. Although considered as the "gold standard", real-time PCR detecting IS*2404* is expensive, sophisticated and unable to confirm viability of bacteria (Sakyi, Aboagye, Darko Otchere, & Yeboah-Manu, 2016). Similarly, culture and histopathology require sophisticated laboratory space, equipment and expertise, whereas microscopy is less sensitive despite being simple and cost effective (Sakyi, Aboagye, Darko Otchere, et al., 2016). Thus, development of quick, sensitive, inexpensive, and field applicable diagnostics are priorities for disease management. With this in mind, techniques to diagnose BUD such as loop mediated isothermal amplification (LAMP), chromatographic and aptamer technique to detect mycolactone and serological techniques to detect mycolactone and bacterial proteins are in various stages of development (Sakyi, Aboagye, Darko Otchere, et al., 2016; Sakyi, Aboagye, Otchere, et al., 2016). LAMP technique detects *M. ulcerans* DNA with

modest equipment requirement, however, it is not considered field applicable at its current stage of development as there are challenges with DNA extraction and maintenance of isothermal conditions that must be optimized (Beissner, Phillips, et al., 2015; Röltgen, Cruz, Ndung'u, & Pluschke, 2019). Similarly, chromatographic techniques such as fluorescence-based thin layer chromatography detecting mycolactone can be impacted by the presence of other co-extracted human lipids (Röltgen et al., 2019). Serological tests for detecting antibodies can be useful for determining seroprevalence of *M. ulcerans* exposure in populations, however, challenges of cross-reactivity among mycobacterial species and inability to distinguish between healthy controls and BUD patients still remains (Röltgen et al., 2019). ELISA based techniques detecting mycolactone and the bacterial protein MUL_3720 as antigens have shown high specificity, however the sensitivity of these tests needs to be further improved (Röltgen et al., 2019). Furthermore, immune-based assays to detect specific T cell subsets have also been proposed as potential diagnostic tools (Nausch et al., 2017).

Prevention of BUD is challenging owing to its unknown mode of transmission. However, studies have shown that use of bath soap, full-body clothing during agricultural activities and proper management of wounds and insect bites are protective against BUD (N'krumah et al., 2016). Despite unclear preventive strategies, an effective treatment of the disease at an earlier stage prevents the disability and deformities in patients. The standard treatment regimen for BUD recommended by the WHO includes rifampicin (10mg/kg body weight daily) and and clarithromycin (7.5 mg/kg per body weight twice daily) (World Health Organization, 2020b).. In Australia, either fluoroquinolones or clarithromycin are used along with rifampicin for the treatment (World Health Organization, 2012b). Surgery (debridement, skin grafting and scar

revision) is also often performed as a complimentary treatment for large lesions (World Health Organization, 2012b).

1.7 Socio-cultural, psychological and economic impact of the disease

Buruli ulcer disease often has a social-cultural, psychological and economic impact on the affected populations. The disease has been perceived to be associated with witchcraft or evil and ancestral spirits by some local populations (Koka et al., 2016). Buruli ulcer patients are thus, often stigmatized within their community (Ahorlu, Koka, Kumordzi, Yeboah-Manu, & Ampadu, 2013; Koka et al., 2016). Loss or disruption of school for up to one year has also been reported (Amoakoh & Aikins, 2013). Further, a study in Ghana reported beliefs that having sex and wound exposure to other people negatively impacted healing (Koka et al., 2016). It has also been shown that former BUD patients sometimes have difficulty in employment, and often feel marginalized in other social aspects related to meeting people, giving opinions, sustained relationships or participating in recreational activities (de Zeeuw et al., 2014). Buruli ulcer patients have sometimes faced social isolation due to common beliefs of the disease being contagious (Aujoulat, Johnson, Zinsou, Guédénon, & Portaels, 2003). Moreover, misrepresentation of BUD patients as being unhygienic by communication media has fueled further stigmatization (Ahorlu et al., 2013). Scarring, especially following surgical treatment, has been shown to cause reduced self-esteem and problems with getting married or continuation of marriage (Aujoulat et al., 2003). And financial burden and fear of contracting disease has also been shown to cause divorce in married couples (Stienstra, Van Der Graaf, Asamoa, & Van Der Werf, 2002). Overall, studies show that BUD patients in some areas are perceived with negative attitudes in the society and possess significant impact to the affected populations (de Zeeuw et al., 2014).

The psychological impact of BUD has been mainly contributed to factors such as disfigurement, physical pain following surgery, financial burden during treatment, self-dissatisfaction for not fulfilling gender roles and stigma and marginalization (Agbenorku, 2011; Hofstraat & Van Brakel, 2015; Ribera, Grietens, Toomer, & Hausmann-Muela, 2009). It has been shown that BUD patients are sometimes abandoned during treatment at clinics due to stigma and financial burden which leads to loneliness, humiliation, insecurity and hopelessness (Ribera et al., 2009). Apart from stigmatization by society, internalized stigma leads to reduced individual self-esteem, and has been reported to evoke embarrassment and fear towards social interactions (Hofstraat & Van Brakel, 2015). Disfigurement, especially facial scarring following surgery, has been reported to precipitate inferiority complex, timidity and lowered self-esteem in reporting patients (Agbenorku, 2011). One study showed that some patients feel unattractive, infertile, incapable of leadership, rejected and considered less in the society (Stienstra et al., 2002). Furthermore, anxiety among patients regarding disease progression and probability of subsequent amputation is common (Ackumey, Gyapong, Pappoe, Kwakye-Maclean, & Weiss, 2012). And, long hospital-stays and physical pain following surgery add to patient stress and trauma (Constantine, Julius, Forbin, & Matchawe, 2011).

In West Africa, disease occurs in impoverished populations, who often live far from health facilities, or who are dependent on farming, fishing or other jobs that require a daily working schedule in the natural environment. Hence, disease can yield a large economic burden due to disruption of their livelihood and earnings, as well as cost for travel and hospital stay (Ackumey, Gyapong, Pappoe, Maclean, & Weiss, 2012). A Ghana study conducted in 2012 found that BUD imposes an annual average cost of US $570 to the affected household, which accounted for nearly 17% of per capita income in the same year (Amoakoh & Aikins, 2013).

Although antibiotics for BUD treatment is free when offered and available, expenses are mainly due to travel and food cost, compelling the affected household to lend money, or sell assets or agricultural fields to cover the cost, imparting further poverty (Ackumey, Gyapong, Pappoe, Maclean, et al., 2012; Amoakoh & Aikins, 2013). Long-term disabilities inflicted by delayed BUD treatment can deprive patients from school and employment opportunities, hampering income generation in those populations who are largely dependent on physical labor, thereby increasing economic inequality in the society (Andrés Garchitorena et al., 2015). Even in developed Australia, a significant economic burden ($14,000/ patient and estimated collective cost of $2,548,000 in Victoria in 2016) is imposed by BUD due to expensive antibiotics which are sometimes not covered by health system's schemes, and with morbidity that can eventually require plastic surgery (O'Brien, Athan, Blasdell, & De Barro, 2018). Severe morbidity of BUD clearly has significant economic impacts in affected households and communities.

1.8 Microbiology of Buruli ulcer disease

Buruli ulcer disease is caused by the slow growing environmental pathogen, *M. ulcerans.* *M. ulcerans* is an acid-fast bacterium in the same genus as *M. tuberculosis* and *M. leprae* (Phylogenetic analyses showing evolutionary relationship of *M. ulcerans* with other mycobacteria is shown in Figure 1.2). The bacterium can grow at temperatures ranging from 29-33^{0}C (World Health Organization, 2019a). It grows better under laboratory conditions aerobically, or microaerophilicly in liquid media such as the BACTEC system (Palomino, Obiang, Realini, Meyers, & Portaels, 1998). *M. ulcerans* has a doubling time between 24 to 84 hours, depending on growth conditions, and as a result, visible growth can take from 4-6 weeks, and up to 9-12 months (Mark E. Benbow et al., n.d.; F Portaels et al., 2001). The colony characteristics of *M. ulcerans* depend on the strains, and often appear as rough and yellow-

pigmented colonies for African strains, with only slight pigmentation for Australian strains, and non-chromogenic colonies for some South American strains (Guerra Laso et al., 2019; F Portaels et al., 2001). Similarly, colony characteristics of *M. ulcerans* subsp. *Shinshuense* responsible for BUD in Japan and China are variable as rough colonies with yellow pigmentation have been reported from Japan whereas pale cream colonies have been reported from a patient acquiring the disease in China (Nakanaga, Hoshino, Yotsu, Makino, & Ishii, 2011; K. Suzuki et al., 2019). The bacterium is unable to grow at 37^0C, thus explaining its limitation to cause only localized infections (Mark E. Benbow et al., n.d.).

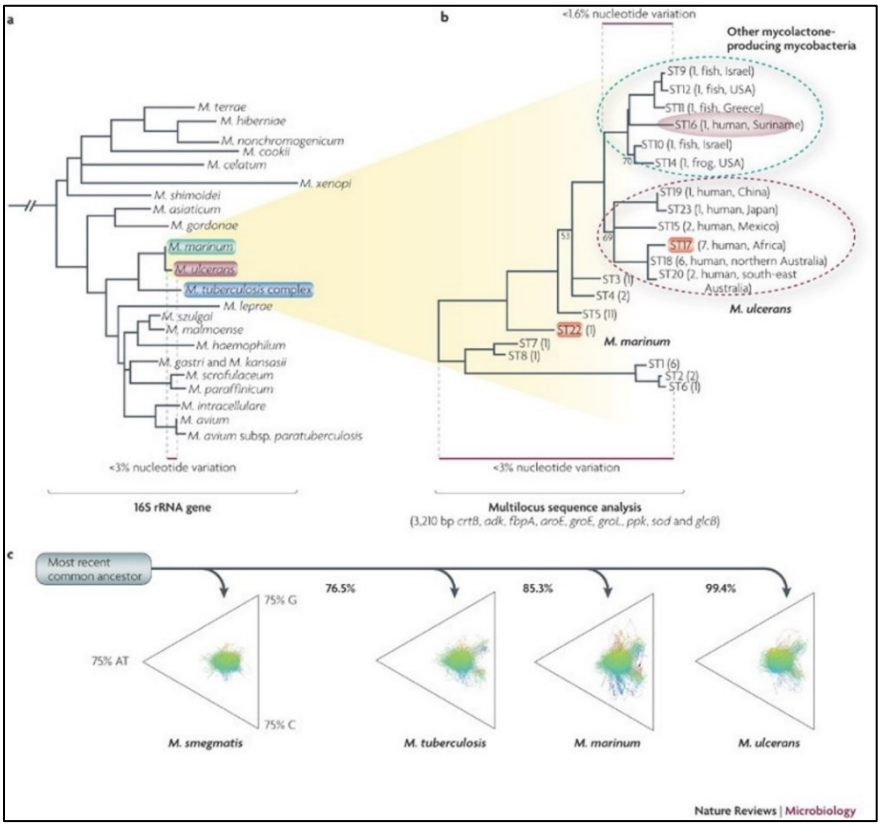

Figure 1.2 Phylogenetic analyses showing evolutionary relationship of *M. ulcerans* with other mycobacteria.

This image was obtained from paper by Demangel et al. (2009) published on Nature Reviews Microbiology(Demangel, Stinear, & Cole, 2009).

1.9 Pathogenicity and virulence

An ulcer with undermined edges occurs in BUD due to sloughing of enlarged fat cells, covering skin and underlying tissues (Rondini et al., 2006). The histopathology of BUD is characterized by necrosis, epidermal hyperplasia and granulomas with minimal inflammation (Guarner et al., 2003). Phillips *et al.* (2014) observed defective circulating T-lymphocytes and a decrease in serum proteins and inflammatory mediators in BUD patients compared to controls (Phillips, Sarfo, et al., 2014). The clinical feature of BUD is mainly attributed to mycolactone produced by *M. ulcerans* (George et al., 1999). Mycolactone was first isolated in 1999 by George *et al.* and was so named as 'mycolactone' to reflect its mycobacterial origin and toxin structure (George et al., 1999). This polyketide lipid cytotoxin is the only known major virulence factor, and consists of a conserved 12-membered lactone ring with unsaturated fatty acid side chains that can vary among different congeners of mycolactone (Kishi, 2011). Among these congeners, mycolactone A/B, C and D are produced by *M. ulcerans*, whereas mycolactone E and F are produced by the *M. ulcerans* ecovars *M. liflandii* (frog pathogen) and *M. pseudoshottsii* (fish pathogen), respectively (Figure 1.3). Mycolactone F is also produced by another fish pathogen, mycolactone-producing *M. marinum* (MPMM) (Kishi, 2011; Ranger et al., 2006). Mycolactone congeners have different carbon backbone lengths (mycolactone A/B, C and D have hexadecenoic acid backbones whereas mycolactone E and F have pentadecanoic backbones) and degrees of unsaturation (Figure 1.3) (Kishi, 2011). Mycolactone congeners have differing degrees of cytotoxicity, and also vary geographically. For instance, Mve-Obiang *et al.* (2003) reported mycolactone A/B being the most cytotoxic of all congeners, and as the major mycolactone produced by African strains. However, mycolactones C and D are the major

mycolactone congeners produced by Australian and Asian strains respectively (Mve-Obiang, Lee, Portaels, & Small, 2003).

Genes for mycolactone A/B production are encoded on the 174 Kb plasmid, pMUM001, which consists of polyketide synthase genes responsible for mycolactone production (Timothy P. Stinear, Pryor, Porter, & Cole, 2005). Genes *mls*A1 and *mls*A2 synthesize the mycolactone core, *mls*B synthesizes the side chains and other accessory genes, and *mup*045 and *mup*038 synthesizes accessory proteins required for joining of subunits (Fred Stephen Sarfo et al., 2016). Variations in the presence or absence in pMUM001 genes yields the congeners mycolactone C and D (Pidot et al., 2008).

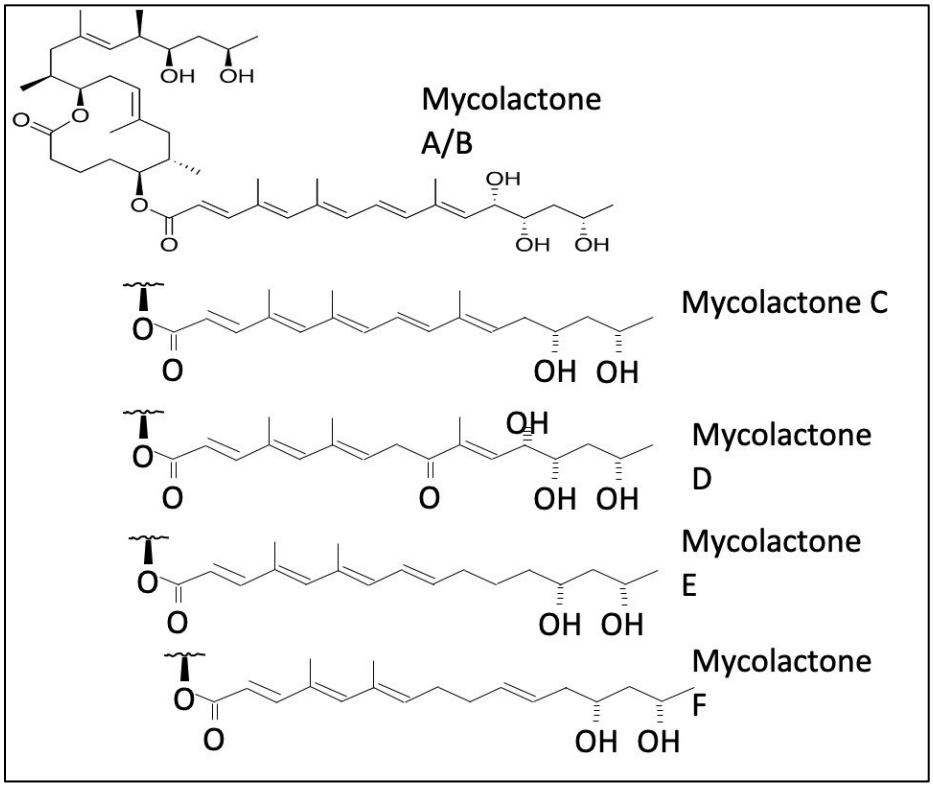

Figure 1.3 Structural comparison of mycolactone congeners.

All congeners share the 12 membered macrolide core structure. But, variations in the southern chain yield congeners that are geographically distributed, and with varying degrees of virulence.

Ability to produce mycolactone differentiates *M. ulcerans* from classical *M. marinum,* a fish pathogen with whom it shares 99.6% sequence homology (T. P. Stinear, Jenkin, Johnson, & Davies, 2000; Timothy P. Stinear, Hong, et al., 2005). *M. ulcerans* is thought to share a common ancestor of classical *M. marinum* and with genome reduction and the acquisition of the mycolactone plasmid evolved into an environmental pathogen with documented genomic and toxin geographic diversity (Figure 1.3). Plasmid pMUM001 is suggested to be acquired by *M. ulcerans* at an expense of extensive gene loss during evolution from its progenitor *M. marinum* thereby suggesting its significant role in adaptation to a new environmental niche (Yip et al., 2007). Other mycolactone producing mycobacteria have similar plasmids that encode genes for mycolactone production (Tobias et al., 2013) .

It is suggested that *M. ulcerans* and its ecovars evolved from a common *M. marunum* progenitor and consists of 3 main lineages that includes isolates from different species (frog, fish and humans) and geographical locations (South America, Japan, China, Australia and Africa) (Doig et al., 2012; Vandelannoote, Eddyani, Buultjens, & Stinear, 2019). Lineage 1 consists of isolates from frog, fish and humans (South America), lineage 2 consists of human isolates from Japan and China, and lineage 3 consists of human isolates from Australia and Africa (Doig et al., 2012; Vandelannoote et al., 2019). Comparative genomic analysis suggests that *M. ulcerans* in lineage 3 have undergone further reductive evolution and may occupy a restricted niche (Doig et al., 2012). Hence further genomic analysis and comparison to MPMs lineages of *M. ulcerans* isolated from these sources will provide insights on their role in transmission to human host.

Mycolactone is responsible for BUD pathology. Studies have shown that mycolactone-negative *M. ulcerans* were not able to establish disease; however, the ulcer was established upon chemical complementation of mycolactone (Adusumilli et al., 2005). Similarly, intradermal

injection of mycolactone to a guinea pig model established disease, showing mycolactone as a key player in BUD pathology (George et al., 1999). Buruli ulcer disease is painless despite extensive skin damage due to mycolactone-mediated immunosuppression and neuron hyperpolarization (Marion, Song, et al., 2014; Fred Stephen Sarfo et al., 2016). Mycolactone directly binds to Wiskott–Aldrich syndrome protein (WASP) and Sec61 thereby preventing cytoskeleton formation and co-translational translocation of proteins, respectively (Fred Stephen Sarfo et al., 2016). Further, mycolactone causes immunomodulation by preventing maturation, migration and agranulocytes-chemoattractant productions in dendritic cells (Coutanceau et al., 2007). Mycolactone-mediated cytopathicity and apoptosis of macrophages and dendritic cells have been observed (Coutanceau et al., 2007; George, Pascopella, Welty, & Small, 2000). Additionally, mycolactone binds to angiotensin II (AT_2) receptors to hyperpolarize neuron (Song et al., 2017).

While the role of mycolactone in Buruli ulcer pathology is well documented, the function of mycolactone for *M. ulcerans* in natural environments is lacking. The macrolide structure of mycolactone suggests the possibility that the molecule may be an antagonist to bacteria with quorum sensing (QS) machinery (similar to acyl homoserine lactones) or may serve as a regulator of secondary metabolism. Indeed, we have preliminary data showing that mycolactone is antagonistic to quorum sensing in the QS model organism, *Vibrio harveyii*, and also in the opportunistic pathogen, *Staphylococcus aureus* (manuscript in preparation, personal communication, H. Jordan). Below we review the literature related to the detection of *M. ulcerans* in the environment and how doing so has given insight into the ecology of this pathogen, including potential reservoirs and hypothesized vectors, and postulated modes of transmission (Table 2). We argue that it is important to move beyond clinical studies of

pathogenesis and treatment to investigate the biology and ecology of *M. ulcerans* (and other environmental bacteria) in the environment to implement preventive and control strategies associated with natural resources management and within the One Health context.

1.10 The ecology of *M. ulcerans*

Buruli ulcer was first described by Sir Albert Cook in 1897 and the first *M. ulcerans* culture was obtained by MacCallum in 1948 (Wansbrough-Jones & Phillips, 2006). However, despite over a century of discovery, a consensus reservoir and mode of transmission of BUD remains unknown, hence gaining its name as a 'mysterious disease' (Roltgen, K and Pluschke, 2015). Nevertheless, many research efforts have provided significant information regarding risk factors, bacterial burden in the environment and possible modes of transmission. The disease has been shown to associate with aquatic habitats impacted by different forms of landscape disturbance (e.g., gold mining, deforestation) (R. W. Merritt et al., 2010) and over seasons related to weather events and climate patterns (Landier et al., 2015, 2014; van Ravensway et al., 2012). Exposure to water during daily activities such as farming, swimming, fishing and conducting household chores enhance BUD risk (N'krumah et al., 2016). Higher disease burden is observed in areas of heterogenous topography and in close proximity to rivers, wetlands and cultivations suggesting a significant association of BUD with aquatic environments and local irrigation (Landier et al., 2014; Wagner, Benbow, Brenden, et al., 2008). Given these epidemiological associations, it is necessary to better understand how populations of *M. ulcerans* vary among habitats, geographic areas and through seasons.

1.10.1 Detection of *M. ulcerans* in the environment

Being an environmental pathogen, *M. ulcerans* transmission and infection requires the combination of a favorable growth environment for the pathogen, specific contact factors of a host with the pathogen, and specific host factors, all of which occur in natural habitats. These bacteria normally reside in the environment where abiotic factors such as oxygen and pH, and biotic factors such as availability of nutrient sources likely modulate proliferation and mycolactone production (Deshayes et al., 2013; Palomino et al., 1998; Sanhueza, Chevillon, Bouzinbi, Godreuil, & Guégan, 2018; Sanhueza et al., 2016; Sanhueza, Guégan, Jordan, & Chevillon, 2019). Furthermore, human activities associated with the environment are thought to provide contact with *M. ulcerans* in the environment, leading to increased risk of transmission and resulting infection.

The most reliable technique to detect *M. ulcerans* in the environment is quantitative PCR, which targets the presence of the IS*2404* gene followed by detection of enoyl reductase (ER) or another gene present in plasmid pMUM001 (Williamson et al., 2012), such as the keto-reductase (KR) gene. The IS*2404* gene may be present in other environmental mycobacteria along with *M. ulcerans*, and qPCR targeting IS*2404* may be a sensitive but not specific initial screen for detecting *M. ulcerans* in the environment (J. A M Fyfe et al., 2007). Several groups have used a multiplexed PCR technique developed by Fyfe *et al.* (2007) that targets an additional keto-reductase gene present in pMUM01 plasmid and Ct difference between an insertion sequence IS*2404* and IS*2606* to differentiate *M. ulcerans* from other mycolactone producing mycobacteria in the environment (J. A M Fyfe et al., 2007; Maman et al., 2018; Röltgen, Pluschke, Johnson, & Fyfe, 2017; A. Singh, McBride, Govan, Pearson, & Ritchie, 2019; Vandelannoote et al., 2010). Furthermore, other groups have successfully targeted variable number tandem repeats to

differentiate environmental and clinical samples (Williamson et al., 2012, 2008, 2014). Currently, qPCR detection is limited by its inability to detect viability of bacteria; however, to our knowledge, only one instance of cultured *M. ulcerans* from the environment has been reported, as environmental contamination of samples and longer incubation time of *M. ulcerans* causes culture difficulty (Françoise Portaels et al., 2008). Employing newer methodology, such as molecular viability assays that take advantage of photoactivation of intercalating agents to differentiate between live and dead organisms would allow for detection of viable *M. ulcerans* organisms in aquatic habitats, and better determination of *M. ulcerans* replication and niche partitioning in these environments.

1.10.2 Presence of *M. ulcerans* in aquatic environmental samples

Presence of *M. ulcerans* detected by PCR in the environment was first reported by Ross *et al.* 1997, from samples of a swamp and a golf course irrigation system within a BUD outbreak area on Philip Island in Victoria, Australia (Ross et al., 1997). The presence of *M. ulcerans* has since been detected in various environmental samples such as invertebrates, water filtrand (e.g., suspended material in the water column), aquatic plant biofilms and soils in Ghana, Benin, Cameroon, French Guiana and Australia by several research groups (J. A M Fyfe et al., 2007; Kotlowski et al., 2004; Vandelannoote et al., 2010; Williamson et al., 2012, 2008). And presence of *M. ulcerans* was positively correlated with BUD prevalence in Benin (Williamson et al., 2012). Similarly, *M. ulcerans* was detected in the surface biofilms of several plant species in Ashanti and Accra regions of Ghana (McIntosh et al., 2014) and recently associated with the plant rhizosphere and some soils in Cote d'Ivoire(Hammoudi et al., 2020). Marsollier *et al.* (2004) detected *M. ulcerans* identical to clinical strains in aquatic plants, which upon culture into BACTEC medium and subsequent inoculation into mice caused BUD (Marsollier et al., 2004).

In Zio and Yoto districts of Togo, Maman *et al.* (2018) detected *M. ulcerans* in environmental samples such as plants, water and soil with genetic profile similar to clinical *M. ulcerans* (Maman et al., 2018). In Cote d'Ivoire, significant *M. ulcerans* positivity was observed in environmental samples such as water, soils and animal excreta from BUD endemic sites compared to non-endemic sites (Hammoudi et al., 2020; Tian, Niamké, Tissot-Dupont, & Drancourt, 2016). In South America, *M. ulcerans* was detected in stagnant water bodies (Morris et al., 2014). Despite no reports of BUD cases in United States, Hennigan *et al.* (2013) targeted *IS2404* and sequenced for *M. ulcerans* specific protein coding sequences and detected *M. ulcerans* in plant and water samples from Louisiana (Hennigan, Myers, & Ferris, 2013), demonstrating the wide distribution of *M. ulcerans* across various geographical locations and matrices. Other *M. ulcerans* ecovars that do not cause human disease, but rather, disease in fish or frogs should also be considered in the latter study, as the study targeted only three loci present in *M. ulcerans* (Hennigan et al., 2013). And, the presence of *M. ulcerans* in the environment in instances with no BUD cases underscores the role of human activities and contact in disease transmission. These collective findings also suggest that *M. ulcerans* is persistent in aquatic environments and is likely a resident member of the associated microbial communities. Understanding the ecology of *M. ulcerans* in multiple habitat types and ecosystems is becoming increasingly recognized as an important step in resolving transmission and developing prevention strategies.

1.10.3 *M. ulcerans* in non-mosquito aquatic invertebrates

In 1999, Portaels *et al.* detected *M. ulcerans* in the insect families Naucoridae and Belostomatidae (both Hemiptera), aggressive aquatic predators that can fly and bite humans, thereby becoming the first to propose the role of aquatic invertebrates as reservoirs and potential

vectors of *M. ulcerans* to humans (Françoise Portaels, Elsen, Guimaraes-Peres, Fonteyne, & Meyers, 1999). Following this finding, several field and experimental investigations were conducted to understand the role of these invertebrates in BUD transmission. *M. ulcerans* was cultured from salivary tissues of these insects collected in Cote d' Ivoire by Marsollier *et al.* (Marsollier et al., 2002). Similarly, Portaels *et al* (2008) were able to successfully culture *M. ulcerans* from another hemipteran group of insects (*Gerris* spp) indicating the presence of live bacteria in these water bugs (Françoise Portaels et al., 2008). *M. ulcerans* was detected in water bugs in several other studies conducted by Williamson *et al.* (2008) and Zogo *et al.* (2015) suggesting a potential role of these water bugs in BUD transmission (Williamson et al., 2008; Zogo et al., 2015). However, a study by Benbow *et al* (2008) from Ghana showed no significant differences in Hemipteran abundance or *M. ulcerans* positivity between BUD endemic and nonendemic sites, nor between abundances of biting Hemipterans and *M. ulcerans* positivity (M. Eric Benbow et al., 2008). Marion *et al.* (2010) found *M. ulcerans* in saliva of water bugs in only endemic regions of Cameroon, which upon inoculation into mice developed Buruli ulcer lesions suggesting presence of live bacteria in insect's saliva (Marion et al., 2010). Also, mathematical modelling by Roche *et al*. (2013) showed that removing Oligochaeta in community network models decreased *M. ulcerans* prevalence in an aquatic community (Roche et al., 2013), suggesting the aquatic food web structure may be important to mediating *M. ulcerans* in the environment. Benbow *et al* (2014) found that Pleidae, Gerridae, Hydracarina and Libellulidae were significant indicator taxa in lotic habitats positive for IS*2404* and ER (Eric Benbow et al., 2014). Similarly, Garchitorena *et al.* (2014) observed that insects of orders Lepidoptera and Hemiptera had higher *M. ulcerans* positivity, with *M. ulcerans* being detected in Hemiptera throughout 11 months in a year (Andrés Garchitorena et al., 2014). Further, In a large spatial

scale of over 90 sites in Ghana, Benbow *et al.* (2014) showed that macroinvertebrate communities significantly differed and had higher abundance of piercing predators in IS*2404* and ER-positive sites compared to ER-negative sites in lotic habitats (Eric Benbow et al., 2014). Furthermore, In Japan, *M ulcerans subsp shinshuense* was present in crayfish within a backyard of a house whose multiple household members had ulcers (Ohtsuka et al., 2014). Taken together, these previous studies demonstrate that *M. ulcerans* is consistently associated with aquatic macroinvertebrate communities, represented by both biting and non-biting species, and that the relative composition of some species compared to others (i.e., food web structure) may play an important role in controlling its abundance in nature.

1.10.4 *M. ulcerans* in mosquitoes

In Australia, mosquitoes are considered *M. ulcerans* vectors, where studies have shown *M. ulcerans* in mosquitoes (Paul D.R. Johnson et al., 2007; Lavender et al., 2011). Moreover, Lavender *et al.* observed that BUD occurrence correlated with *M. ulcerans* positivity in wild-caught mosquitoes (Lavender et al., 2011), and documented that patients reported BUD lesions at mosquito bite sites. Risk analysis also showed that use of insect repellant reduced risk of BUD, suggesting a role of mosquito in pathogen transmission (Lavender et al., 2011; Quek et al., 2007). In Victoria, BUD cases were reported in areas where other mosquito transmitted diseases such as Ross River virus (RRV) and Barmah Forest virus (BFV) infections were reported (P. D R Johnson & Lavender, 2009). Furthermore, Sanders *et al* (2017) demonstrated higher attraction and oviposition behavior of mosquitoes to a site containing higher mycolactone, thereby suggesting a role of mycolactone in interkingdom signaling between *M. ulcerans* and mosquitoes (Sanders et al., 2017). Landier *et al.* (2011) reported that using bed nets decreased risk of BUD in Cameroon (Landier et al., 2011). In contrast, Zogo *et al.* (2015) (Zogo et al., 2015) found no

M. ulcerans positivity in mosquitoes collected in Benin. Similarly, Djouaka *et al.* (2017) detected presence of other IS*2404* positive mycobacterial species, but not *M. ulcerans* in mosquitoes collected in Benin (Djouaka et al., 2017). The discrepancy in *M. ulcerans* detection in mosquitoes in Africa and Australia is unclear and requires further investigation. Since mosquito larvae are part of some aquatic food webs, particularly in slow moving water, it is plausible that adult mosquitoes are simply indicators of a highly *M. ulcerans* contaminated water source and play a minor role in transmission. Given that there is not one scientifically documented case of a bacteria pathogen being transmitted by mosquitoes, the role of these vectors of viruses, protists and worms in transmitting *M. ulcerans* will require additional scrutiny and evidence as discussed by Merritt *et al*. (2010) for BUD (R. W. Merritt et al., 2010).

1.10.5 *M. ulcerans* in mammals

Various studies have reported BUD lesions as well as presence of *M. ulcerans* in feces of animals thereby suggesting a potential zoonotic mode of transmission. In Australia, Fyfe *et al.* (2010) and Carson *et al.* (2013) reported higher presence of *M. ulcerans* in feces of possums in endemic regions that were identical to the clinical strains present in BUD patients (Carson et al., 2014; Janet A.M. Fyfe et al., 2010). Similarly, Lavender *et al*. (2008) detected *M. ulcerans* in marsupial feces that were identical to the Victorian outbreak strain (Lavender et al., 2008). Also, BUD infection ranging from asymptomatic gut colonization to complicated forms such as systemic BUD or BUD lesions of the face and sensitive organs has been reported in ringtail, brushtail and mountain brushtail possums in Australia by O'Brien *et al* (2014) (C. R. O'Brien et al., 2014), and Röltgen *et al.* (2017) observed *M. ulcerans* in bandicoot feces (Röltgen et al., 2017). Similarly, BUD lesions were observed in the tail of a mouse in a Ghana community (Narh et al., 2015). Tian *et al.* (2016) detected *M. ulcerans* in feces of *Thryonomys swinderianus*

(common name is grasscutter), a mammal that lives nearby water resources and rice fields in Cote d'Ivoire and other geographic locations (Hammoudi et al., 2020; Tian et al., 2016). A study by Hammoudi *et al*. (2019) found similar DNA sequence patterns in spleen and rectal content of *T. swinderianus* in Côte d'Ivoire (Hammoudi, Dizoe, et al., 2019). The asymptomatic colonization of *M. ulcerans* in animal guts is intriguing and requires further confirmation on bacterial viability inside the gut. While *M. ulcerans* has been detected, to our knowledge, no studies have isolated viable *M. ulcerans* from animal guts. Additionally, investigations on effects of conditions such as higher temperature and anaerobic environments that might impact *M. ulcerans* survival, growth and mycolactone production can provide further insights on *M. ulcerans* pathology inside the gut. And the interaction of *M. ulcerans* with other intestinal bacteria needs to be conducted.

Lesions characteristic of BUD (Type 1), and positive for *M. ulcerans* were reported in domestic animals such as goats and dogs in Benin (Djouaka, Rousseau Zeukeng et al., 2018), but not in rodents and shrews by Durnez *et al.* (2010) (Durnez et al., 2010). Similarly, *M. ulcerans* was not present in feces of domestic animals in Ghana (Tobias et al., 2016). There have also been reports of characteristics lesions or *M. ulcerans* presence in other mammals such as koalas, horses, alpacas, and cats (A. Singh, McBride, Govan, & Pearson, 2018).

1.10.6 *M. ulcerans* detected in other sources

Several other taxa such as fish and amphibians have been reported to harbor *M. ulcerans* DNA. Wilson et al. (2012) detected *M. ulcerans* in Leptopelis frogs (Willson et al., 2013). Similarly, *M. ulcerans* infection was observed in the Indian flap-shelled turtle by Sakaguchi et al. (2011) in Japan (Sakaguchi et al., 2011). *M. ulcerans* in the study by Wilson *et al.* (2012) was confirmed by VNTR analysis and in Sakaguchi *et al* and by PCR amplification of IS*2404*

followed by sequence analysis on BLAST in the NCBI gene bank (Sakaguchi et al., 2011; Willson et al., 2013). Further, BUD following a snake bite was described by Hofer et al. (1993); however, a possibility of disease in this case could also be mechanical inoculation of *M. ulcerans* rather than the snake serving as reservoir of the bacteria (Hofer et al., 1993).

Despite speculation of an animal reservoir or zoonotic transmission, human-human transmission of disease has been shown to be unlikely. O'Brien *et al*. (2017) observed that no genetic relatedness occurred in *M. ulcerans* infecting different members in a family, and Sarfo *et al.* (2011) did not find *M. ulcerans* in feces of BUD patients, suggesting that humans may not play any role as a reservoir or in transmission of BUD (O'Brien et al., 2017; Fred S. Sarfo et al., 2011). However, BUD induced by human bite, suggesting a potential mechanical role, has been reported (Martine Debacker, Zinsou, Aguiar, Meyers, & Portaels, 2003).

1.11 Hypothesized modes of transmission

Given the high diversity of potential hosts among animals and habitats, it is clear that *M. ulcerans* is in various environments and in a range of abundances. However, how *M. ulcerans* is transmitted to humans is much less understood, with several groups proposing different modes of transmission, and a few suggesting complex, multiple modes of transmission that may vary geographically and with environmental type.

1.11.1 Transmission through skin trauma

One of the earliest hypothesized modes of transmission was that of sharp plants cutting the host and *M. ulcerans* being inoculated directly from the plant or subsequently with exposure to nearby soil into the existing wound (Barker, Clancey, Morrow, & Rao, 1970; Barker, Clancey, & Rao, 1972; Revill & Barker, 1972; Stanford & Paul, 1973). At the time, it was not possible to

culture *M. ulcerans* or other slow growing mycobacteria from environmental samples, but there was anecdotal evidence that some environmental mycobacteria from plants, including *M. ulcerans*, could be associated with patient isolates; however, *M. ulcerans* was never confirmed (D. Barker et al., 1970). In the 1990s, there was a reported association of a BUD outbreak with a spray irrigation system in Australia that led to the hypothesis that *M. ulcerans* transmission occurred via aerosols introduced to the skin through injuries or infection to pre-existing cuts or wounds (Ross et al., 1997; Veitch et al., 1997). However, this hypothesis of aerosolized transmission was not supported later, based on genomic analysis which suggested that reductive evolution of *M. ulcerans* and loss of various gene functions make bacteria unlikely to be free-living in the environment (Paul D.R. Johnson et al., 2007; Timothy P. Stinear et al., 2007). In 1999, Walsh *et al.* showed that intradermal inoculation of *M. ulcerans* led to BUD in armadillos (Walsh, Meyers, Krieg, & Walsh, 1999). Using a hairless guinea pig model, Williamson *et al.* (2014) demonstrated the inability of *M. ulcerans* to cause infection of a passively inoculated abrasion site; however, infection was established upon subdermal injection of *M. ulcerans.* This study was one of the first to establish that puncture is necessary for inoculation and establishing BUD pathology under controlled laboratory conditions (Williamson et al., 2014). Wallace *et al.* (2017) showed that minor puncture (<2 mm deep) or mosquito bite of *M. ulcerans* contaminated skin can cause mechanical transmission of BUD (Wallace et al., 2017). Azumah *et al.* (2017) reported that BUD was established when *M. ulcerans* was topically applied to puncture at a 1.5mm depth suggesting the possibility of passive *M. ulcerans* transmission in a pre-established deep wound or puncture (Azumah et al., 2017). Collectively, these experiments demonstrate that although *M. ulcerans* may not be inoculated via passive infection through superficial cuts and wounds, its inoculation underneath the skin either through mechanical injury (anthropogenic,

environmental puncture or mosquito bites) or post-contamination of *M. ulcerans* on previously established deep puncture may lead to transmission and BUD pathogenesis.

1.11.2 Amoebae

M. ulcerans is considered fastidious and its reduced genome suggests a specific niche environment. *M. ulcerans* has been postulated to persist in aerosolized amoebic cysts where amoeba such as *Acanthamoeba* spp have been shown to harbor other pathogens such as *M. marinum, M. avium* and *Legionella pneumophila* thereby aiding in transmission of these bacteria (Amissah et al., 2014; Wilson, Boakye, Mosi, & Asiedu, 2011). The co-cultivation of *M. ulcerans* with amoeba such as *A. polyphaga* was shown to demonstrate intracellular survival for 2 weeks; however, there was a log CFU *M. ulcerans* decrease (Gryseels et al., 2012). Similarly, Amissah *et al.* (2014) reported very low *M. ulcerans* survival within *A. castellanii* at 28 days thereby suggesting a possible role of amoeba as a host but less likely in transmission(Amissah et al., 2014). In the study, there was presence of IS*2404* positive DNA in 4.64% of amoebae cultivated from environmental sources; however, they were all negative for additional targets of IS*2606* and KR-B genes (Amissah et al., 2014). In contrast, Azumah *et al*. (2017) observed *M. ulcerans* viability in association with *A. polyphaga* for up to 42 days. However, it was unclear from that study whether *M. ulcerans* replication was measured. Topical inoculation of *M. ulcerans*-*A. polyphaga* culture to mouse footpads led to earlier inflammation compared to *M. ulcerans* injections alone (Azumah et al., 2017). While these laboratory studies suggest a role for amoebae as reservoirs, more research is needed regarding intracellular survival and replication of *M. ulcerans* in amoebae in natural environments.

1.11.3 Aquatic invertebrates

As discussed earlier, several studies have showed aquatic invertebrates harbor *M. ulcerans* in the environment. However, the potential of non-adult mosquito invertebrates, such as biting water bugs, to transmit *M. ulcerans* is questionable. To address this question, several laboratory experiments were conducted to study *M. ulcerans* transmission via biting water bugs (Hemiptera). Marsollier *et al*. (2002) observed that experimental infection of *M. ulcerans* to Naucoridae localizes bacteria to its salivary gland, and bites led to BUD infection in a mice model (Marsollier et al., 2002). Further, Mosi *et al.* (2008) demonstrated that an African *M. ulcerans* isolate could colonize Belostomatidae, including the head, raptorial arms, thorax, salivary glands and gut; and then could be transferred to blow fly larvae (Diptera: Calliphoridae) (*Phormia regina*) during feeding (Mosi, Williamson, Wallace, Merritt, & Small, 2008). Both Marsollier *et al*. (2002) and Mosi *et al*. (2008) observed no clinical effect of *M. ulcerans* to invertebrates, and Marsollier *et al*. (2002) further demonstrated inability of other targeted mycobacterium species to colonize these insects, thereby suggesting potential adaptation of *M. ulcerans* as a commensal (Marsollier et al., 2002; Mosi et al., 2008). Moreover, Marsollier *et al*. (2005) also demonstrated experimentally that only mycolactone producing *M. ulcerans* (but not mycolactone-mutant *M. ulcerans* or *M. marinum*) were able to colonize salivary glands of the aquatic insect *Naucoris cimicoides* and biting of mice by these insects developed non-ulcerative and oedamatic BUD in mice (Marsollier et al., 2005). Similarly, inoculation of *M. ulcerans* positive saliva derived from Belostomatidae caused BUD lesions in mice (Marion et al., 2010). A case report of BUD lesions following a belostomatid bite was reported by Marion *et al.* (2014) (Marion, Chauty, et al., 2014). However, the potential of aquatic invertebrate vectors remains controversial as Naucoridae and Belostomatidae are not hematophagous and do not actively seek

and attack humans; but, the chances of accidental biting and mechanical transmission by these water bugs in response to disturbance in their habitat still remains a possibility worth additional investigation (Marsollier et al., 2002; Mosi et al., 2008). Furthermore, bivalves, snails and other invertebrates that do not bite or otherwise physically injure humans or other animals have been shown to harbor *M. ulcerans* in numbers similar to mosquitoes, piercing insects and vertebrate feces (M. Eric Benbow et al., 2008; Eric Benbow et al., 2014; R. W. Merritt et al., 2010).

1.11.4 Mosquitoes

Mosquitoes are one of the postulated modes of *M. ulcerans* transmission in Australia, where several studies have demonstrated *M. ulcerans* DNA in adult mosquitoes. As previously mentioned, a laboratory study by Wallace *et al.* (2017) showed that minor puncture (<2 mm deep) or mosquito bite of *M. ulcerans* contaminated skin can cause mechanical transmission of BUD (Wallace et al., 2017). Tobias *et al*. (2009) showed that *Aedes camptorhynhcus* larvae can feed on possum excreta contaminated with *M. ulcerans*, which can colonize and sustain inside the mosquito larva gut and mouthparts (Tobias et al., 2009). Similarly, Wallace *et al*. (2010) showed that *M. ulcerans* was sustained inside mosquito larvae as well as in salivary glands and guts of Belostomatidae that fed on the larvae. However, *M. ulcerans* was not maintained at pupae and adult stages and adult mosquitoes were not able to mechanically transmit the bacteria (Wallace et al., 2010). Djouaka *et al.* (2017) observed decreased *M. ulcerans* survival during development of larvae from the first to fourth instar stages, and no *M. ulcerans* was detected in adults (Djouaka et al., 2017). Although Wallace *et al.* (2010) showed no developmental defects in *M. ulcerans* contaminated mosquito larvae, Hoxmeier *et al.* (2015) did report decreased survival and developmental defects in *Anopheles gambiae* (Hoxmeier et al., 2015; Wallace et al., 2010). The developmental defect occurred due to disruption of lipid metabolism, a common

feature observed in other pathogens such as *M. tuberculosis* and *M. leprae* (Hoxmeier et al., 2015). The discrepancy in the results could be contributed by different species of mosquitoes used in the study as well as study design and analyses.

Several postulated modes of transmission, research supporting them, challenges to confirm them as mode of transmission and future research directions are provided in table 1.2.

1.12 Importance of quality assurance for determining *M. ulcerans* reservoir

As we have discussed in earlier sections, *M. ulcerans* has been detected in different environmental samples, however, mysteries about its reservoir(s) remains unsolved. *M. ulcerans* is a slow-growing environmental pathogen, hence it is very difficult to cultivate them reliably from environmental samples. Because of this, most of the researchess relies on PCR for the detection of *M. ulcerans* DNA in environmental samples. However, PCR results cannot confirm bacterial viability. Additionally, the technique is prone to contamination and may yield false positive results. Further, it can also yield false negative results due to low *M. ulcerans* DNA concentration and, presence of PCR inhibitors and DNA from other sources (Eddyani et al., 2014). A review of 22 papers that investigated presence of *M. ulcerans* in more than 1 type of environmental sample matrix showed that all these studies performed either PCR or Real Time PCR for detection of *M. ulcerans* (Aboagye et al., 2017, 2016; Bratschi et al., 2014; Combe et al., 2019; Dassi et al., 2017; Eddyani et al., 2008; J. A M Fyfe et al., 2007; A Garchitorena et al., 2015; Hammoudi et al., 2020; Hennigan et al., 2013; Maman et al., 2018; Marsollier et al., 2004; McIntosh et al., 2014; Morris et al., 2014; Narh et al., 2015; Pileggi et al., 2017; T. Stinear et al., 2000; Tano, Dassi, Mosi, Koussémon, & Bonfoh, 2017; Tian et al., 2016; Vandelannoote et al., 2010; Williamson et al., 2012, 2008). The PCR targeted insertion sequences IS*2404* and IS*2606* , and either ketoreductase and enoyl reductase domains on the plasmid responsible for

mycolactone synthesis. Some of the studies confirmed the presence of *M. ulcerans* DNA by either VNTR typing using 3-4 loci or sequencing (Table A.1). But despite all of these, only one study successfully cultivated pure *M. ulcerans* culture from soil and moss (Aboagye et al., 2016). In one of the studies, inoculation of BACTEC culture of an IS*2404* positive environmental sample caused Buruli ulcer lesion. However, the culture was contaminated by *M. szulgai* (Marsollier et al., 2004).

M. ulcerans DNA was detected in various samples such as soil, animal excreta, plant, water, biofilm, detritus, fungi, moss, sediment and invertebrates (including mosquitoes). In most of the studies, *M. ulcerans* positivity was higher in BUD endemic regions compared to non-endemic regions (Hammoudi et al., 2020; McIntosh et al., 2014; Pileggi et al., 2017; Tian et al., 2016; Williamson et al., 2012). Although in one of the studies BUD endemicity did not predict *M. ulcerans* positivity, this could have been contributed by passive surveillance in the location (Williamson et al., 2008). Interestingly in most of the studies, *M. ulcerans* was detected in all types of samples that were tested. However, there was one study where no IS*2404* positive samples were detected in water and biofilm samples in both endemic and non-endemic locations is Benin (Eddyani et al., 2008). In the other study, presence of *M. ulcerans* was detected in only 1 water sample in BU endemic locations in Ghana (Vandelannoote et al., 2010). The reasons for these discrepancies are unclear, however, may be contributed by several factors such as sample type, numbers, locations, seasons, etc. Additionally, there can be presence of other mycolactone producing mycobacteria in the environment that can give false positivity for *M. ulcerans* that causes human disease. Alternatively, these variations could also be contributed by poor quality assurance of laboratories. Multicenter External Quality Assessment by Eddyani *et al.* (2014) showed great variation in laboratories performance on molecular detection of *M. ulcearns*

containing clinical and environmental samples (Eddyani et al., 2014). In this study, few samples were correctly identified by all laboratories, and only two laboratories correctly identified all samples, raising concerns over reliability of PCR data for clinical interpretation and environmental studies (Eddyani et al., 2014). Thus, strict laboratory internal and external quality control and quality assessment is crucial to correctly interpret PCR data that can aid to understand the *M. ulcerans* reservoir and for accurate disease diagnosis presumed infections of humans and animals.

1.13 Challenges and future directions: A perspective on the one-health approach

The data presented above show discrete studies of human cases, pathogenesis, and investigations of environmental reservoirs. But, infection by *M. ulcerans* and progression to BUD involves interaction between human hosts and the pathogen, which depends on the ecological niche of the pathogen, changing environmental conditions that affect the interactions and natural variability in abundance and communities that may mediate exposure. Indeed, aquatic invertebrate communities are used as biomonitors of aquatic ecosystem health and changes due to disturbance. Given the link between ecological disturbance to aquatic water bodies and BUD, invertebrate communities, and the known *M. ulcerans* associations among most taxa, may provide management avenues of disease prevention. Knowledge of the potential of a water source to be at high risk for *M. ulcerans* abundance could be used to mediate the ecological disturbance or warn communities of the risk. BUD prevalence and risk for transmission to occur with resulting pathology is at the nexus of several agents that include landscape and aquatic ecosystem disturbances, pathogen ecological dynamics, food web interactions, human activity and behavior, and individual genetic and host factors. A major challenge in preventing BUD is not having a foundational knowledge of the mode, or modes, of

transmission, in order to implement an effective preventative strategy. Measuring drivers of *M. ulcerans* and mycolactone ecological and evolutionary interactions in natural environments, with humans and with animals in concert that influence environmental persistence, colonization, virulence, and heterogeneity in transmission will allow capture of the first-principle biological processes that generate BUD disease patterns of occurrence in space and time. And to this end, a One-Health approach leading to the comprehensive understanding of the complex interdependences of human, animal, microbial and ecosystem health influencing *M. ulcerans* transmission is a key strategy for breakthroughs in the control, treatment, and prevention of BUD. For this, challenges with institutional capacity, funding, and differences in multidisciplinary perspectives must be overcome to establish a synergistic global network of qualified individuals working locally, regionally, nationally, and internationally. Gaining a better picture of the global problem through enhanced human and environmental surveillance should be an urgent priority, with the goal to prevent exposure and infection, and reduce morbidity.

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Table 1.2 Several postulated modes of transmission, research supporting them, challenges to confirm them as mode of transmission and future research directions.

Postulated Hypothesis	Supporting Research Evidence	Challenges	Future Directions
Amoebae	Survival of *M. ulcerans* inside amoeba demonstrated by several studies (Amissah et al., 2014; Gryseels et al., 2012) Earlier inflammation in mouse footpad observed for topical inoculation of *M. ulcerans-A. polyphaga* culture compared to *M. ulcerans* alone (Azumah et al., 2017)	Replication of *M. ulcerans* inside amoebae not confirmed Study on field research showed no presence of *M. ulcerans* in amoebae cultured from environmental samples (Amissah et al., 2014)	More field investigations Investigations of replication of *M. ulcerans* inside amoebae needs to be confirmed
Aquatic Invertebrates	*M. ulcerans* detected in several aquatic invertebrate taxa (M. Eric Benbow et al., 2008; Eric Benbow et al., 2014; Andrés Garchitorena et al., 2014; Marion et al., 2010; Marsollier et al., 2002; Françoise Portaels et al., 1999, 2008; Williamson et al., 2008; Zogo et al., 2015) Successful culture of *M. ulcerans* from Hemiptera (*Gerris* spp) and salivary tissues of insects (Françoise Portaels et al., 2008) Higher abundance of piercing predators in ER+ sites compared to ER- sites in lotic habitats) (Eric Benbow et al., 2014) Inoculation of *M. ulcerans* found in saliva of water bugs developed Buruli ulcer lesion in mice (Marsollier et al., 2002) Experimental infection showing colonization of *M. ulcerans* to salivary gland and other organs such as head, raptorial arms, thorax and guts (Marsollier et al., 2002; Mosi et al., 2008) Development of BUD infection in mice upon biting by experimentally infected Naucoridae (Marion et al., 2010) A case report of BUD lesions following Belostomatidae bite reported (Marion, Chauty, et al., 2014)	Role of aquatic invertebrates in BUD transmission still controversial as these aquatic invertebrates do not normally bite humans. There have been no published confirmations of laboratory experimental infections of aquatic invertebrates. Bivalves, snails and other invertebrates that do not bite or otherwise physically injure humans or other animals have been shown to harbor *M. ulcerans* in numbers similar to mosquitoes, piercing insects and vertebrate feces.	Field studies investigating presence of biting insects in BUD affected sites and presence of *M. ulcerans* in them Investigations into ability of *M. ulcerans* replication in these insects Investigations into role of these insects in mechanical transmission of BUD

Table 1.2 (continued)

Postulated Hypothesis	Supporting Research Evidence	Challenges	Future Directions
Mosquitoes	Presence of *M. ulcerans* in mosquitoes in Australia (Paul D.R. Johnson et al., 2007; Lavender et al., 2011) Insect repellant, bed nets decreased BUD risk (Landier et al., 2011; Lavender et al., 2011; Quek et al., 2007) BUD reported in areas with other mosquito transmitted diseases (P. D R Johnson & Lavender, 2009) Higher attraction and oviposition of mosquitoes in mycolactone containing sites (Sanders et al., 2017) Mosquito larvae can feed on possum excreta contaminated with *M. ulcerans*, which can colonize and sustain inside larva's gut and mouthparts (Tobias et al., 2009) *M. ulcerans* sustained inside mosquito larvae as well as in salivary gland and gut of Belostomatidae that fed on the larvae (Wallace et al., 2010)	No *M. ulcerans* detected in mosquitoes in Africa (Djouaka et al., 2017; Zogo et al., 2015) Although *M. ulcerans* sustained inside mosquito larvae as well as in salivary gland and gut of Belostomatidae that fed on the larvae, *M. ulcerans* was not maintained at pupae and adult stages and adult mosquitoes were not able to mechanically transmit the bacteria (Wallace et al., 2010) Studies have shown decreased *M. ulcerans* survival during development of larvae from L1-L4 stage, and no *M. ulcerans* was detected in adult stages (Djouaka et al., 2017)	Investigations into role of mosquitoes as a mechanical vector for BUD. Field investigations on presence of *M. ulcerans* in mosquitoes in Africa Investigation into discrepancy regarding the presence of *M. ulcerans* DNA in Australia and Africa.

Table 1.2 (continued)

Postulated Hypothesis	Supporting Research Evidence	Challenges	Future Directions
Marsupials and Mammals	Presence of *M. ulcerans* in feces of mammals and marsupials in Australia (Röltgen et al., 2017) *M. ulcerans* in feces of possums and marsupials that were identical to clinical strains (Carson et al., 2014; Janet A.M. Fyfe et al., 2010; Lavender et al., 2008) BUD lesions in body parts of several mammals such as ringtail, brushtail and mountain brushtail possums, mouse, grass cutter, goats, dogs and other mammals in Australia and Africa (Djouaka, Rousseau Zeukeng et al., 2018; Narh et al., 2015; C. R. O'Brien et al., 2014; A. Singh et al., 2018)	No studies have isolated viable *M. ulcerans* from animal guts. *M. ulcerans* was not detected from Benin in rodents and shrews and *M. ulcerans* was not present in feces of domestic animals in Ghana (Tobias et al., 2016; Vandelannoote et al., 2010)	The asymptomatic colonization of *M. ulcerans* in animal guts is intriguing and requires further investigation regarding effects of anaerobic conditions and temperature, among other conditions that might impact *M. ulcerans* survival, growth and mycolactone production. Further, the interaction of *M. ulcerans* with other intestinal bacteria needs to be conducted.
Human-human (unlikely mode of transmission)	No genetic relatedness occurred in *M. ulcerans* infecting different members in a family (O'Brien et al., 2017) Absence of *M. ulcerans* in feces of BUD patients (Fred S. Sarfo et al., 2011)	BUD induced due to human bite, suggesting more of a mechanical role of human has been reported (Martine Debacker et al., 2003)	-

CHAPTER II

TOXIN PRODUCTION BY ENVIRONMENTAL PATHOGENS: A SURVIVAL STRATEGY AND VIRULENCE CONTRIBUTER

(LITERATURE REVIEW)

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2.1 Introduction

Although penicillin was one of the original "magic bullets" of medical science for targeted bacterial cell death, it is generally assumed that the primary purpose of its production as a secreted secondary metabolite is to provide a fitness advantage to *Penicillium* to allow outcompeting or defense against other microbes in its natural environment (Bajaj, Veiga, Van Dissel, Pronk, & Daran, 2014). In fact, Flemming's original discovery of penicillin was unintentionally based on interactions between a contaminating *Penicillium* strain with *Staphylococcus* variants on agar plates (Flemming A., 1929). Penicillin's mechanism of action once secreted, is binding and inhibition of the transpeptidase that catalyzes the final step in the cross-linking of peptidoglycan, an essential and conserved component of bacterial cell walls

(Fernandes, Amador, & Prudêncio, 2013). This is an example of how a microbe produces a secondary metabolite outside of a host, with significant ecological function, to increase the microbe's survival ability; and in this case, the function also provides a medical tool for treating infectious agents. But many of these molecules do not have therapeutic value, and in other contexts, may even increase virulence during infection in susceptible hosts.

A fluctuating environment influences the overall microbial community that can significantly affect genetic exchange, metabolic diversity and expression of genes and metabolites that ultimately lead to pathogenicity and virulence in a host, and there is accumulating evidence that biotic interactions in the environment can play a vital role in preadaptation and evolution of microbial pathogens (Metcalf et al., 2015; Moënne-Loccoz, Mavingui, Combes, Normand, & Steinberg, 2015; Sironi, Cagliani, Forni, & Clerici, 2015). Furthermore, species within larger microbial communities have differential gene expression and production of small molecules, peptides, and proteins in response to abiotic conditions such as dynamic oxygen, pH, and salinity conditions. Changes in abiotic variables may push a microbial species to a "niche edge", where the organism may have reduced survival or reproduction; they must adapt or die (Brooks, Turkarslan, Beer, Yin Lo, & Baliga, 2011; Cantrell, Dianese, Fell, Gunde-Cimerman, & Zalar, 2011; Pekkonen, Ketola, & Laakso, 2013).

Environmental heterogeneity thus promotes species distribution variability and divergence that directly impacts evolutionary trajectories toward nonpathogenic or pathogenic species and subspecies (Percy, 2007). It seems unlikely that the microbe would expend energy necessary to synthesize complex molecules such as toxins, if they offered it no advantage, and pathogenic traits that are acquired should thus be favored by natural selection to help organisms to acquire nutrients, colonize, proliferate and increase diversity, and spread in natural

environments; but then may yield symptoms in the susceptible host. The acquisition of genes associated with the production of toxins and other products likely facilitate niche expansion and adaption, provide a competitive advantage, mediate predation, or some other lifestyle advantage. And, in more general terms, the environmental lifestyle can attenuate or strengthen virulence once in or on a host, depending on the underlying biology of the system.

The coincidental evolution of virulence hypothesis posits that virulence is not always a direct target of selection, but is sometimes a by-product of microbial evolution in response to selective forces caused by biotic and abiotic factors in the outside-host environment, possibly as microbial weapons (Gould & Lewontin, 1979; B. R. Levin, 1996)(Figure 2.1). Compared to life in the within-host environment, environmental microbes are directly exposed to a complexity of environmental, biotic and abiotic interactions during their free-living life stage. And, it is selection in a microbe's normal life cycle in the environment during inter- (and intra-) domain and species competition within a community context that leads to production of "toxins" or other molecules, evolution with a host, or a stochastic process. Thus, it is the microbe being at the "wrong place at the wrong time" and being subjected to host immune responses, that leads to pathogenicity and increased virulence in a susceptible host.

These secondary metabolites evolved millions of years before human discovery. And, while the functions of these molecules as toxic compounds in disease may be well understood, the ecological value of these in shaping phenotypes, fitness, and community composition of environmental pathogens in the context of microbial communication, evolution and ecology is under explored. Further, relatively little is known about the environmental signals to which these molecules respond. In this review, we focus on some prokaryotic and eukaryotic, toxin producing environmental pathogens that impact animals and plants. We give an overview of their

niche, epidemiology of resulting diseases, the function of the toxin in pathogenesis, and known or suggested functions in natural environments. We end by discussing the need for ecological theory in hypothesis testing and integration of microbial and ecological data to investigate the roles of these complex molecules in natural environments, in order to better understand heterogeneity in transmission, and to guide disease management efforts.

2.2 Toxin producing bacteria

Bacterial toxins are a major group of virulence factors used by pathogenic microbes promoting infection in the affected host and are classified as either endotoxins or exotoxins. Endotoxins are a structural component (mainly lipopolysaccharides) of gram-negative bacteria such as *Escherichia coli* and *Salmonella* spp that can cause symptoms including fever, inflammation and lethal shock in the host; whereas exotoxins are secreted proteins or enzymes that can affect host organs or systems (Peterson, 1996). Although most toxins are proteins, mycolactone, a cytotoxin produced by *Mycobacterium ulcerans* is a lipid (Peterson, 1996; Fred Stephen Sarfo et al., 2016). Toxins can be encoded in either chromosomes, such as in the cholera toxin for *V. cholerae* or plasmids such as mycolactone in the pMUM001 plasmid (Peterson, 1996; Timothy P. Stinear, Pryor, et al., 2005). Toxin mechanisms can be cytotoxic, enterotoxic or neurotoxic, and can cause diseases ranging from mild symptoms such as vomiting and diarrhea to fatal conditions such as neuroparalytic botulism caused by botulinum toxin (Peterson, 1996; Shukla & Sharma, 2005). Botulinum produced by *Clostridium botulinum* is the most potent known neurotoxin, where one gram can kill over a million people and 39.2 grams has the potential to eradicate humankind (Dhaked, Singh, Singh, & Gupta, 2010; Shukla & Sharma, 2005). These toxins may facilitate bacterial pathogenesis in a susceptible host through

upregulation of other virulence genes; however, in some cases they are the primary virulence factors (Deshayes et al., 2013).

Many bacterial environmental pathogens produce toxic compounds outside of their known hosts, indicating that the evolved function of the toxin may be more than to allow the pathogen to enter and replicate within the host, but that it may also provide a fitness advantage in their natural habitat. For instance, it has been hypothesized that an aquatic bacterium, *Vibrio cholerae*, uses its toxin system to sense nutrient limitations, in defensive mechanisms against predation, for establishment of symbiotic relationships with hosts, and for niche adaptation (Sakib, Reddi, & Almagro-Moreno, 2018).

2.2.1 Aquatic habitats

2.2.1.1 *Vibrio cholerae*

2.2.1.1.1 Taxonomy, Distribution, and Disease

The family Vibrionaceae, order Vibrionales, are a group of gram negative, facultative anaerobes (Farmer, 2006). They are motile with one or more usually polar flagella, contain oxidases and are capable of using fermentation to obtain energy. Many Vibrionaceae species are widely distributed in the environment, where they contribute to the cycling of organic and inorganic compounds. Others may have mutualistic associations with fish and other marine life, or may be pathogenic for fish, eels, and frogs, as well as other vertebrates and invertebrates. *Vibrio cholerae* are one of over 160 species within the genus *Vibrio, and* are halophilic, curved bacteria that are commonly found in brackish river, estuarine and coastal waters, either in a planktonic state or attached to biotic and abiotic surfaces, zooplankton, cyanobacteria and phytoplankton (ALMAGRO-MORENO & TAYLOR, 2013). *V. cholerae* is the causative agent

of cholera, an intestinal disease characterized by profuse rice-watery diarrhea, muscle cramps and dehydration that can lead to renal necrosis, stroke and even death in severe cases (Somboonwit, Menezes, Holt, Sinnott, & Shapshak, 2017). Despite being less prevalent in developed countries where there is access to good water sanitation facilities, the global impact of cholera is huge with around 1.3 billion people at risk across 69 endemic countries that cause an estimated 2.86 million cases and 95,000 death annually (Ali, Nelson, Lopez, & Sack, 2015). *V. cholerae* O1 and O139 are two strains mainly responsible for recent and past outbreaks (World Health Organization, 2019c).

2.2.1.1.2 Pathogenesis

Infection occurs primarily due to ingestion of contaminated food and water and is mainly prevalent in areas with poor water sanitation (ALMAGRO-MORENO & TAYLOR, 2013). A high infectious dose (10^3- 10^8 cells) is required to cause disease and symptoms can appear within 2 hours to 5 days post infection (Schmid-Hempel & Frank, 2007; Somboonwit et al., 2017). Pathogenesis is due largely to a bipartite toxin produced by *V. cholerae*, known as cholera toxin (CT) that binds to the intestinal epithelial cells and releases cyclic AMP leading to massive loss of chloride and water, diarrhea and dehydration (Matson, Withey, & DiRita, 2007). Upon entry into the human small intestine, the *V. cholerae* ToxR regulon is activated that upregulates expression of various virulence factors including CT and toxin co-regulated pilus (a type IV pilus that helps in colonization) contributing to *V. cholerae* pathogenesis (Renee Bina, Taylor, Vikram, Ante, & Bina, 2013). Biofilm formation and detachment mediated by quorum sensing mechanisms, along with flagellar motility facilitates bacterial attachment, survival and spread inside the human host (Silva & Benitez, 2016). In addition to CT, *V. cholerae* secretes other toxins such as cholix toxin, hemolytic exotoxin called cytolysin (VCC), and multifunctional

autoprocessing repeats-in-toxin (MARTXvc) that damage cellular function, induce inflammatory activity and enhance colonization, and facilitate immune evasion, respectively (Ou et al., 2009; Sakib et al., 2018).

2.2.1.1.3 Toxin Production in the Environmental Context

Although these toxins and virulence factors contribute to pathogenicity in humans, pathogenic strains have largely evolved from nonpathogenic environmental strains through horizontal transfer of virulence-related gene clusters (Faruque et al., 1998; Faruque & Mekalanos, 2003). *V. cholerae* have evolved broad niche specialization and is exposed to a wide range of abiotic and biotic stressors that pose threats to its survival, such as nutrient limitations, pH changes, temperature, pressure, and salinity fluctuations, and protozoan and phage predation. Thus, genes for toxin production are assumed to have been a preadaptation to enhance survival of *V. cholerae* in the natural environment by sensing nutrient concentrations, promoting adherence and motility, colonization and establishment of symbiotic relationships to copepods and chironomids, and other natural reservoirs. A study by Islam (1990) showed increased toxin production by *V. cholerae* O1 during survival with the cyanobacterium, *Rhizoclonium fontanum*, though the biological significance of this has yet to be resolved (Islam, 1990). Further, ToxR production is regulated *in vitro* by nutrient limitation, alkaline pH, and in response to amino acids asparagine, arginine, glutamate and serine (Mey, Butz, & Payne, 2015; Mey, Craig, & Payne, 2012; Reen, Almagro-Moreno, Ussery, & Boyd, 2006; Sakib et al., 2018). Additionally, these toxins confer cytotoxicity and bacteriocin-like activity to protect the producing bacteria against predators and provide a competitive advantage against incompatible *Vibrio* strains and other microbes (Sakib et al., 2018; Unterweger et al., 2014). For instance, it was shown that *V. cholerae* strains can kill each other in a Type VI secretion system-dependent manner, and strains

can be subdivided into compatible strains that are able to coexist and incompatible strains that outcompete and kill each other, where toxigenic *V. cholerae* strains cluster within a single group of compatible strains (Unterweger et al., 2014).

2.2.1.2 *Vibrio vulnificus*

2.2.1.2.1 Taxonomy, Distribution, and Disease

V. vulnificus are halophilic, gram-negative, rod-shaped bacteria that are part of the natural flora of coastal marine environments such as estuaries and brackish ponds, and in association with species such as fish, oysters, clams, shrimp and shellfish (Jones & Oliver, 2009). *V. vulnificus* is a heterogeneous species that comprises virulent and avirulent strains associated with multiple hosts (Hernández-Cabanyero et al., 2020). Further, *V. vulnificus* can cause a wide variety of infections ranging from gastroenteritis to severe sepsis and skin and soft tissue infections (Horseman & Surani, 2011; K. C. Wong, Brown, Luscombe, Wong, & Mendis, 2015). Transmission can occur orally by consumption of raw or uncooked seafood (most commonly oysters) or through direct contact of contaminated water on pre-formed wounds, injuries or puncture wounds caused by contaminated sharp objects or marine life (Klontz et al., 1988; OLIVER, 2005). Symptoms associated with acute gastroenteritis caused by *V. vulnificus* include diarrhea, cramps, nausea, vomiting, fever and chills. However, invasive sepsis can occur in immune deficient patients including those with chronic liver disease, to whom it can cause severe symptoms and death (Horseman & Surani, 2011). The clinical feature for wound infection can range from mild to severe, with severe infections characterized by local cutaneous lesions (Horseman & Surani, 2011; Jones & Oliver, 2009). *V. vulnificas* can be classified into three different biotypes, where biotype I is primarily responsible for human infection, biotype II

includes primarily eel pathogens, and biotype III includes both human and freshwater fish pathogens, with limited geographical range (Heng et al., 2017).

2.2.1.2.2 Pathogenesis

V. vulnificus has several virulence factors contributing to adherence (e.g. pili and flagella), survival inside the human host (e.g. production of decarboxylase and superoxide dismutase to escape low stomach pH, capsular polysaccharide to evade immune system, siderophores and heme receptors for iron acquisition) and pathology [e.g. toxins ($MARTX_{Vv}$, hemolysin (Vvha), and metalloprotease (VvPE), lipopolysaccharide and membrane proteins (OmpU and IlpA)] (Horseman & Surani, 2011). MARTXvv is similar to that in *V. cholerae*, and forms pores to lyse host cells; however, its effect is not limited to a direct pathological effect as it contributes to pathogenesis by preventing phagocytosis and increasing paracellular permeability to facilitate colonization, intracellular survival and dissemination to distal organs (Gavin, Beubier, & Satchell, 2017; Hor & Chen, 2013; Lo et al., 2011). Similar to MARTXvv toxin, Vvha toxin forms pores on host cells. But loss of the toxin does not change *V. vulnificus* pathogenicity in a mouse model (Wright & Morris, 1991).

2.2.1.2.3 Toxin Production in the Environmental Context

The two most important environmental factors that control *Vibrio* dynamics in aquatic environments are temperature and salinity (Kelly, 1982). *V. vulnificus* is detected in sea water and on crustacea generally only in summer months, when temperature is at least 17°C, and increases in global climate change are suggested to influence the distribution (Deeb, Tufford, Scott, Moore, & Dow, 2018). Decrease in water temperature below 10^0C causes cell numbers to drop below detectable levels, reaching the viable but non-culturable state (VBNC), which can

become metabolically active again with increased temperature (Strom & Paranjpye, 2000). Also, environmental temperature drives virulent phenotypes where strains have been found to be highly virulent in hosts at 28°C but avirulent at or below 20°C (Hernández-Cabanyero et al., 2020; Tilton & Ryan, 1987). A study of twenty-four clinical and environmental isolates showed no difference in virulence between the isolates in a mouse model (Stelma, Reyes, Peeler, Johnson, & Spaulding, 1992). Interestingly, another study found that there was widespread DNA polymorphisms between clinical and environmental isolates (Chatzidaki-Livanis, Hubbard, Gordon, Harwood, & Wright, 2006). Pathogenicity islands were also identified in an environmentally isolated *V. vulnificus* strain, suggesting that environmental strains could serve as reservoirs for virulence genes (Klein, Pipes, & Lovell, 2018).

Kim, et al (2006) showed that *vvhA* expression was decreased during swarming and after deletion of the AI-2 quorum sensing system, suggesting this toxin may play a greater role in environmental survival than in human infection. The authors suggest *vvh*A may be involved in differentiation from swarming to planktonic phenotypes (M. Y. Kim et al., 2006). MARTXvv toxin and other *V. vulnificus* toxins were also suggested to promote environmental fitness through biofilm formation on chitin, adherence to aquatic flora, or to resist predation (Klein et al., 2018; Rahman et al., 2008).

2.2.1.3 *Legionella pneumophila*

2.2.1.3.1 Taxonomy, Distribution, and Disease

Legionella is the only genus with the family Legionellaceae, order Legionellales ("Genus: Legionella," n.d.). *L. pneumophilia* is the etiological agent of Legionnaires' disease and, less commonly, Pontiac Fever (Correia et al., 2016). While 65 different species of *Legionella* exist, many of which are pathogenic, *L. pneumophilia* accounts for 90% of cases of

Legionnaires' disease (Newton, Ang, Van Driel, & Hartland, 2010). The inhalation of small water droplets contaminated with *L. pneumophilia* and subsequent colonization of the respiratory tract leads to symptoms such as fever, cough, chills, and/or muscle aches (Newton et al., 2010). *L. pneumophilia* is a gram-negative rod-shaped bacterium naturally found near and within freshwater environments, though it can also be found in man-made areas including air conditioning cooling towers, plumbing systems, and hot water tanks (Newton et al., 2010; Steinert, Hentschel, & Hacker, 2002; Winn, 1985).

2.2.1.3.2 Pathogenesis

Pathogenesis is largely due to the ability of *L. pneumophila* to invade and grow within alveolar macrophages, and it is widely believed that this ability resulted from preadaptation to intracellular niches in its natural environment (Boamah, Zhou, Ensminger, & O'Connor, 2017). Protein secretion is also critical, and Type II and Type IV secretion systems are necessary for *L. pneumophila pathogenesis*. The Type II system secretes a collection of degradative enzymes required for growth at lower temperatures, colony morphology, intracellular infection of amoebae, macrophages and monocytes, and virulence in a murine model of pneumonia. The type IV system likely exports effector proteins critical for phagosome trafficking. *L. pneumophilia* also produces a toxin belonging to the Rtx family ("repeats-in toxin") that is involved in adherence, cytotoxicity, and pore formation. The *rtxA* gene encodes the *dot*/*icm*-regulated pore-forming toxin, plays a significant role in the entry of *L. pneumophilia* into host cells, and correlates with the ability to cause disease in humans (D'Auria et al., 2008).

2.2.1.3.3 Toxin Production in the Environmental Context

L. pneumophilia are ubiquitous organisms in freshwater environments, and can survive a wide range of conditions including pHs between 2.7 and 8.3 (Anand, Skinner, Malic, & Kurtz, 1983; Sheehan, Henson, & Ferris, 2005) and temperatures between 35 °C and 70°C (Dennis, Green, & Jones, 1984; Wadowsky, Wolford, McNamara, & Yee, 1985). A recent study found that Type II protein secretion is also critical for growth at 12 to 25°C but not at 30 to 37°C. Type II secretion mutants also showed reduced survival in both tap water at 4 to 17°C and aquatic amoebae at 22 to 25°C, correlating low-temperature survival and secretion function among tested *Legionella* species. While *Legionella* are sometimes found free-living within aquatic environments, they are more commonly found within mixed community biofilms, or replicating intracellularly within protozoa (Boamah et al., 2017). *L. pneumophilia's* relationship with protozoa further protects it from harsh environmental conditions in both natural and artificial environments. This bacterium can be ingested by many free-living amoebae, creating a parasitic or mutualistic relationship between *L. pneumophilia* and the amoeba (Atlas, 1999), protecting the bacterium from biocides, thermal disinfection (J. Barker, Brown, Collier, Farrell, & Gilbert, 1992; Storey, Ashbolt, & Stenström, 2004), and potentially acting as a "training ground" for survival inside eukaryotic cells, including human macrophages (Belyi & Aktories, 2010; Molmeret, Horn, Wagner, Santic, & Kwaik, 2005). Studies of the *L. pneumophilia* Type II secretion system have identified over 20 Type II dependent proteins within the secretome, including novel proteins, aminopeptidases, an RNase, and chitinase, that contribute to differential infection in amoebae (Debroy, Dao, Söderberg, Rossier, & Cianciotto, 2006; Söderberg, Dao, Starkenburg, & Cianciotto, 2008; Tyson et al., 2013).

Despite its role in disease, strains with the *rtx* locus were found in 95% of isolates from environmental water sources in Greece (Katsiaflaka et al., 2016). Another study found *rtx*A present in 8 of 14 *Legionalla* human and environmental isolates. But in contrast to data from previous work in murine and human macrophages, wild-type *L. pneumophila* and a *rtx*A mutant did not produce cytotoxicity or pore formation in *A. castellanii* amoebae (Cirillo, Yan, Littman, Samrakandi, & Cirillo, 2002). The group found *rtx*A involved in adherence, entry, intracellular survival and trafficking in amoebae, allowing persistence within this environmental reservoir. *L pneumophila* also secretes homogentisic acid (HGA), that stimulates *L. pneumophila* growth and iron uptake under iron-limiting conditions (Zheng, Chatfield, Liles, & Cianciotto, 2013). Also, HGA and HGA-melanin can reduce and release iron from both insoluble ferric hydroxide and the mammalian iron chelates ferritin and transferrin. HGA secretion is also hypothesized as a means for reducing competition from inter-*Legionella* strains, and other bacteria present in microbial communities, though specific microbial interactions have not been investigated (T. C. Levin, Goldspiel, & Malik, 2019). Another known defense mechanism of *L. pneumophilia* is the secretion of cytotoxic glycosyltransferases which can inhibit protein synthesis in target cells (Belyi & Aktories, 2010). While the identity and roles of all the extracellular products *L. pneumophilia* secretes have yet to be identified, *L. pneumophilia* can also secrete proteases, lipases, acid phosphatase, deoxyribonucleases, and β- lactamases into their environment (Thorpe & Miller, 1981). Further research will provide a clearer picture of how other toxins secreted as defense mechanisms by *L. pneumophilia* influence the introduction and persistence of *L. pneumophila* in natural environments.

2.2.1.4 *Mycobacterium ulcerans*

2.2.1.4.1 Taxonomy, Distribution, and Disease

There are currently 224 identified species and 21subspecies in the genus *Mycobacterium* not in the *M. tuberculosis* (MTB) complex and more species have been identified within the last 20 years than in the last 114 years total ("Genus: Mycobacterium," n.d.). In spite of the recent profusion of new mycobacterial species, recent reports document that 30% of mycobacterial isolates from water, soil, air, and patients do not belong to any of the identified species. Likely there are numbers of species yet to be discovered, especially in environmental habitats where culture may not always be advantageous (Shahraki, Heidarieh, Hashemzadeh, & Khandan, 2015; Van Ingen et al., 2010). This under-speciation also leaves the numbers of clinically relevant members underappreciated. *Mycobacterium ulcerans* is a non-tuberculosis mycobacteria that is the causative agent of Buruli ulcer, a necrotizing, yet often painless, skin disease prevalent in West Africa, and Australia. Buruli ulcer is endemic in over 33 countries worldwide, and is the third most common mycobacterial infection after tuberculosis and leprosy (Babonneau et al., 2015; Organization, 2016). An exact mode of transmission of *M. ulcerans* to humans has not yet been determined and has involved scientists and researchers for the past seven decades (Tim Stinear, 2014). Several modes of transmission have been proposed including passive transmission via open cuts and wounds during contact with environments containing *M. ulcerans*, bites of aquatic insects or mosquitoes or transmission through aerosols or amoeba (Lavender et al., 2011; Marion et al., 2010; Wilson et al., 2011). Failure to establish a passive infection in abrasion sites in an animal model raised questions regarding routes of transmission, such as whether puncture of *M. ulcerans* contaminated skin would lead to disease pathology, whether the skin microbiome or aquatic microbial consortia affects colonization and infection,

and whether mycolactone is modulated differentially with response to differing routes of infection and local environments (for instance, abrasion versus injection and surface versus subdermal) (Wallace et al., 2017; Williamson et al., 2014).

2.2.1.4.2 Pathogenesis

The main virulence factor of *M. ulcerans* is a cytotoxic lipid compound, mycolactone, whose genes are encoded by a giant plasmid pMUM001. Mycolactone consists of a 12-membered lactone ring with acyl side chains (Fred Stephen Sarfo et al., 2016). Mycolactone is the only toxin known to be associated with a mycobacterium, apart from *M. ulcerans* and its ecological variants, and *M. ulcerans* and mycolactone congeners are conserved within specific geographic locations, with varying degrees of virulence (Daniel, Lee, Portaels, & Small, 2004; Mve-Obiang et al., 2003). The toxin is immunosuppressive and prevents the co-translational translocation of many immune-related proteins that would normally pass through the endoplasmic reticulum for secretion or placement in cell membranes. This mechanism inhibits dendritic cell maturation and migration to the draining lymph node in mice, and inhibition of other immune cells such as T- cells and cytokine production has been observed (Coutanceau et al., 2007; Fred Stephen Sarfo et al., 2016). Further, mycolactone causes hyperactivation of Wiscott Aldrich Syndrome proteins, leading to cytoskeletal rearrangement, and cell cycle arrest within the cell cycle growth phase (Fred Stephen Sarfo et al., 2016).

2.2.1.4.3 Toxin Production in the Environmental Context

The effects of mycolactone on host cells and Buruli ulcer pathology are well documented (B. S. Hall et al., 2014). What is less clear, however, is the role mycolactone serves in fitness for the organism in natural environments, and what the “true target” is. One factor allowing *M.*

ulcerans to thrive in a variety of environments is the ability to colonize plant and invertebrate surfaces and to form biofilms (Kotlowski et al., 2004; Marsollier et al., 2007, 2004; Williamson et al., 2008). Also, a significant positive correlation of higher pH (A Garchitorena et al., 2015), and negative correlation to brackish water and presence of *M. ulcerans* in aquatic habitats has been observed (Asmar, Sassi, Phelippeau, & Drancourt, 2016). Additionally, mycolactone is structurally similar to quorum sensing compounds in other bacteria, suggesting the possibility that mycolactone is a molecule involved with quorum sensing or antagonistic mechanisms (Chakraborty & Kumar, 2019; Sanders et al., 2017). Also, it has been experimentally demonstrated that mycolactone is an attractant for mosquito blood-feeding, and an oviposition stimulant (Sanders et al., 2017). Mycolactone is also regulated by glucose, maltose, and maltopentose, though not at the transcription or translational level (Deshayes et al., 2013). Further, this regulation was linked to upregulation of iron uptake machinery. However, recently, Sanhueza, et al. measured the transcriptome of *M. ulcerans* cultivated in media containing chitin, or chitin and calcium, compared to controls (Sanhueza et al., 2019). They found three plasmid genes associated with mycolactone production expressed without any change in expression level between control and any modified conditions. The authors suggested that lack of mycolactone gene expression may correlate to a lack of a stress response under their growth conditions. Further studies are needed to determine the impact of these and other abiotic and biotic actors, which could be encountered during *M. ulcerans* infection and within its environmental niche, on mycolactone gene expression and production.

2.2.2 Terrestrial habitats

2.2.2.1 *Bacillus cereus*

2.2.2.1.1 Taxonomy, Distribution, and Disease

The *Bacillus cereus* group consists of Gram-positive, rod-shaped, spore-forming aerobic bacteria that are widespread in natural environments. The group includes eleven closely related species: *B. anthracis, B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. cytotoxicus, B. toyonensis, B. gaemokensis, "B. manliponensis" and B. bingmayongensis*. Although these species are differentiated by phenotypic characteristics and pathological properties, genome sequencing data have shown that they are closely related, and share 99% 16s sequence similarity (Y. Liu et al., 2015). Species within the *B. cereus* group are also difficult to distinguish because evolutionary mechanisms driving virulence diversity are not well understood, and differences in temperature tolerance place species into differing phylogenetic clusters. *Bacillus cereus* is a saprophytic spore former that is ubiquitous in nature, though soil is considered the main reservoir (Bottone, 2010). Although *B. cereus* can yield a variety of illnesses such as infections of the respiratory tract, central nervous system, urinary tract, skin and nosocomial infections, *B. cereus* is largely known for foodborne intoxication, mainly associated with consumption of fried rice (S. D. Bennett, Walsh, & Gould, 2013; Bottone, 2010). Ingestion of either toxins, or vegetative cells or spores within contaminated food leads to either emetic or diarrheal forms, respectively (Logan, 2012). The emetic form is caused by preformed toxin, cereulide, that causes nausea and vomiting within a short incubation period (0.5-6h); whereas the diarrheal form occurs as abdominal pain and watery diarrhea at comparatively longer incubation time (8-16h) due to enterotoxin production from vegetative bacteria (Carroll et al., 2019; Logan, 2012). Although food borne illness caused by this

bacterium is self-limiting with appropriate hydration, disease can still progress to cause severe complications that can lead to death in immunocompromised individuals (McDowell; & Friedman, 2019).

2.2.2.1.2 Pathogenesis

Cereulide is resistant to proteolysis, is pH and heat stable, and does not elicit an immune response (Granum & Lund, 1997). Genes encoding cereulide are encoded on a large plasmid, almost exclusively present in a single monomorphic cluster from an evolutionary lineage of closely related strains (Ehling-Schulz et al., 2006, 2005). Cereulide inhibits mitochondrial activity by inhibition of fatty acid oxidation and can lead to liver failure. Enterotoxins produced by the bacterium include non-hemolytic Nhe, and the hemolysin BL (HBL), both pore-forming tripartite toxins acting in a sequential manner to cause diarrhea (Sastalla et al., 2013). Besides, cereulide toxins and enterotoxins, *B. cereus* has proteases, phospholipases and four different hemolysins: I (cereolysin O), II, III and IV (cytotoxin K, or cytK). These pore forming toxins contribute to pathogenesis by lysing host cells to either facilitate spread and nutrient acquisition (hemolysin II) and cause pathology related to diseases such as gastroenteritis (cytK) and endophthalmitis (hemolysin I) (Ramarao & Sanchis, 2013).

2.2.2.1.3 Toxin Production in the Environmental Context

Some reports suggest that some members of the *B. cereus* group do not germinate and grow in soils unless a nutrient source, such as decomposing plants, insects or animals is present (Fricker, Ågren, Segerman, Knutsson, & Ehling-Schulz, 2011; Saile & Koehler, 2006; Vilas-Bôas et al., 2000; West, Burges, Dixon, & Wyborn, 1985). While another report suggested that *B. cereus* may possess an alternative endophytic niche endophytic lifestyle (Ehling-Schulz,

Frenzel, & Gohar, 2015; Hoornstra et al., 2013). *B. cereus* has also been suggested to be an insect symbiont of soil dwelling arthropods, and has been transiently detected in mammalian guts, as well as in soil, plants, and dairy sources. And though not often detected, Østensvik (2004) isolated cytotoxic strains from several riverine sources (Østensvik, From, Heidenreich, O'Sullivan, & Granum, 2004).

B. cereus initiates toxin production only at high population densities, and toxins and putative toxins constitute approximately 32–39% of the secreted proteins during the early exponential growth phase under nutrient limitation (Ceuppens et al., 2011; Clair, Roussi, Armengaud, & Duport, 2010). Thus, toxin production presents a considerable cost to the *B. cereus* cell, which would not be maintained unless it also yielded considerable advantage. While spores and biofilm formation increases fitness, nutrient acquisition and pore formation provides a competitive advantage against other microbes in the human host and also in natural environments (Ehling-Schulz et al., 2015; Gohar et al., 2008). For instance, cereulide toxins are regulated by various external stimuli such as temperature, oxygen, nutrient sources and pH, and displays broad activity against a wide range of fungi (Ladeuze, Lentz, Delbrassinne, Hu, & Mahillon, 2011). Cereulide synthesis occurs between 12 to 37 °C, though some psychrotolerant strains can also produce the toxin. Cereulide producing *B. cereus* also differ from nonemetic strains by starch hydrolysis, haemolysis, and lecithinase reaction. Some data suggest a biological role of cereulide in *B. cereus* adaptation to specific niches and outcompeting fungi and other soil microbes in natural environments (Ehling-Schulz et al., 2015). While others suggest a role as a siderophore, as its expression increases fitness in potassium deprived environments, during growth in alkaline environments, such as in decaying plant material in the soil and the insect gut [15]. Additionally, PlcR, a *B. cereus* transcriptional regulator is part of a quorum sensing system

controlling expression of several enterotoxins, haemolysins, phospholipases and proteases involved in nutrient acquisition, cellular protection, and environmental signaling (Gohar et al., 2008; Stenfors Arnesen, Fagerlund, & Granum, 2008).The production of these toxins under diverse environmental conditions and niches suggests widespread environmental roles.

2.2.2.2 *B. anthracis*

2.2.2.2.1 Taxonomy, Distribution, and Disease

B. anthracis, causative agents of anthrax, are gram positive, spore forming rods that are commonly found in soil (Turnbull, 1996). This species is considered one of the most potent biological weapons and was used as a biological weapon during the first world war (Centers for Disease Control and Prevention, 2017; Spencer, 2003). It is the only obligate *Bacillus* pathogen in vertebrates and one of the first bacteria against which a vaccine was developed (Turnbull, 1996). Anthrax mainly occurs due to direct and indirect contact with infected animals and their products (Centers for Disease Control and Prevention, 2017; Turnbull, 1996). The disease is classified into different forms; cutaneous anthrax, inhalational anthrax, gastrointestinal anthrax and injection anthrax based on the portal of entry and site of infection (Centers for Disease Control and Prevention, 2014; Turnbull, 1996). Cutaneous anthrax, the most common and least dangerous form of anthrax, can occur when spores infect lesions or via flies bite (Centers for Disease Control and Prevention, 2014; Turnbull, 1996). The infection can be self-limiting and heal spontaneously, however, lesions on the face or neck can obstruct airways and cause secondary meningitis and septicemia (Turnbull, 1996). If left untreated, 20% of cutaneous anthrax can be fatal (Centers for Disease Control and Prevention, 2014; Turnbull, 1996). Gastrointestinal anthrax occurs via ingestion of spores and affects gastrointestinal tract, however, can spread to the lymphatic system (Centers for Disease Control and Prevention, 2014; Turnbull,

1996). Inhalation anthrax occurs due to inhalation of spores, which enters the pulmonary system and then spreads throughout the body (Centers for Disease Control and Prevention, 2014; Turnbull, 1996). Due to late diagnosis, this is the most dangerous form of anthrax with approx. 90% mortality in untreated patients and approx. 45% mortality in patients with aggressive treatment (Centers for Disease Control and Prevention, 2014; Turnbull, 1996). Injection anthrax has been reported in drug users and have clinical manifestations similar to cutaneous anthrax.

2.2.2.2.2 Pathogenesis

Major virulence factors of *B. anthracis* are tripartite anthrax toxin and polyglutamate capsule that are encoded in large plasmids pXO1 and pXO2 (Fasanella, 2013; J. Sun & Jacquez, 2016; Turnbull, 1996). The polyglutamate capsule provides immune evasion by protecting against phagocytes whereas anthrax toxin contribute to disease pathology by direct destruction of organ and tissues (Turnbull, 1996). Both anthrax toxin and capsule are needed for full virulence and loss of any one of these factors can largely reduce the virulence potential of bacteria (Fasanella, 2013). Anthrax is an A-B type toxin that consists of protective antigen (PA) as a binding moiety that allow binding and pore formation in the cell and edema factor (EF) and lethal factor (LF) as an enzymatic catalytic moieties that are responsible for characteristic edema and cell destruction respectively (J. Sun & Jacquez, 2016; Turnbull, 1996). The pore formed by PA facilitates entry of EF (calmodulin-dependent adenylate cyclase) and LF (zinc dependent metalloprotease) into the cell (J. Sun & Jacquez, 2016; Turnbull, 1996). Upon entry, EF exhibits its adenylate cyclase activity to raise cAMP levels that cause water retention for the development of characteristic edema whereas LF destroys mitogen-activated protein kinase kinases (MAPKKs) to cause cell death (J. Sun & Jacquez, 2016). Lethal toxin is suggested to be crucial for dissemination and persistence of *B. anthracis* in a host during inhalational anthrax as these

processes were halted in a mutant lacking the toxin in a mouse model (Loving et al., 2009). Further, lethal and edema toxin also aids in virulence by conferring immune evasion during infection to the host (Loving et al., 2009).

2.2.2.2.3 Toxin Production in the Environmental Context

B. anthracis toxin production and sporulation are inversely related and *in vivo* bicarbonate and CO_2 rich environmental conditions increases toxin production and decrease sporulation whereas vice versa is observed in an environment external to the host (Dale, Raynor, Ty, Hadjifrangiskou, & Koehler, 2018). This alteration in the bacterium's lifestyle is an important survival strategy to evade host immune response during its life inside the host and protect against harsh environments during its life outside the host (Dale et al., 2018). It is also suggested that *B. anthracis* infects its host and exhibits excessive virulence so that it can germinate into vegetative cells to multiply within the host and then kill the host to release spores into the environment, a strategy to proliferate and maintain its existence (Fasanella, 2013). In fact, another interesting field experiment showed that *B. anthracis* spores increased the rate of establishment of a native grass by 50% and that grass seeds exposed to zebra blood reached heights that were 45% taller than controls (Ganz et al., 2014). They also detected significant effects of the grass and *B. anthracis* on taxon richness and bacterial community composition. The authors suggest that interactions between *B. anthracis* and plants may result in increased host grazing for dispersal and subsequently increased transmission to hosts (Ganz et al., 2014).

2.2.2.3 *Clostridium botulinum*

2.2.2.3.1 Taxonomy, Distribution, and Disease

Members of the genus *Clostridium* are classified by being gram positive (though sometimes reacting as gram negative), spore formers that utilizes anaerobic metabolism and can carry out dissimilatory sulfate reduction (Andreesen, Bahl, & Gottschalk, 1989). There are currently 265 validly published strains and the genus is one of the largest genera among prokaryotes ("Genus: Clostridium," n.d.). *Clostridium botulinum* is a species that produces the most lethal substance known, botulinum toxin (BoNT) (World health organization, 2018). They are ubiquitous and have been found in soil, intestinal tracts of mammals and sediments in aquatic habitats (Espelund & Klaveness, 2014; World health organization, 2018). *C. botulinum* causes illnesses either due to intoxication or infection (World health organization, 2018). Food-borne botulism is an intoxication that occurs due to ingestion of preformed toxins present in food (World health organization, 2018). Infections due to *C. botulinum* such as infant botulism, wound botulism and inhalational botulism occur due to infection of spores that germinate and release toxins in the site of infection (World health organization, 2018). *C. botulinum* are divided into different groups (I-IV) and produce different toxin serotypes (A-H) (Espelund & Klaveness, 2014). Group I are proteolytic strains whereas group II includes non-proteolytic strains of *C. botulinum* (Espelund & Klaveness, 2014). Botulinum toxin serotypes produced by group I (A, B and F) and II (B, E and F) cause botulism in humans (Espelund & Klaveness, 2014; World health organization, 2018). Toxin serotype A followed by B and F are considered the most potent BoNT (Nigam & Nigam, 2010). The mortality rate of foodborne botulism is high (5-10% of cases) (Chellapandi & Prisilla, 2018; World health organization, 2018). Types C and D are associated with animal infection, particularly waterfowl. Industrial formulations of BoNT such

as Myoblock® and Botox® are also used for pharmaceutical and cosmetic purposes (Abdela, Desta, & Melaku, 2016).

2.2.2.3.2 Pathogenesis

Botulinum is a neurotoxin and affects the nervous system. It blocks release of acetylcholine, a principle neurotransmitter to cause descending flaccid paralysis, which results in respiratory arrest and death (Abdela et al., 2016; Nigam & Nigam, 2010; World health organization, 2018). *C. botulinum* possesses various virulence factors such as adherence proteins (e.g. fbp, groEL), enzymes to protect against oxidative stress (e.g. rubrerythrin, superoxide dismutase and superoxide reductase), toxins (e.g. α-Clostripain, BoNT, Hemolysin A and collagenase) and metabolic regulations that contribute to adherence, immune evasion, nutrient acquisition, survival and disease pathology inside the host (Chellapandi & Prisilla, 2018). Alpha-clostripain is a protease and is involved in posttranslational modification of neurotoxin (Sebaihia et al., 2007). Botulinolysin (BLY), a hemolysin toxin facilitates absorption of botulinum toxin and nutrient acquisition in the intestine (T. Suzuki et al., 2016).

2.2.2.3.3 Toxin Production in the Ecological Context

The primary purpose of some of these virulence factors such as adhesins, cytolysins and proteases is to assist the bacterium's saprophytic life style, however can contribute to attachment and tissue destruction during infection to hosts (Chellapandi & Prisilla, 2018). Additionally, BoNT is suggested to have evolved from a collagenase-like gene cluster and could also play a role in binding and degradation of tissues to support the bacterium's saprophytic life cycle (Doxey, Lynch, Müller, Meiering, & McConkey, 2008). It is suggested that host environmental and nutrient factors impact BoNT production (Chellapandi & Prisilla, 2018; Connan et al., 2012;

Cooksley et al., 2010). BoNT toxin production has been shown to be enhanced by a CO_2 environment in non-proteolytic *C. Botulinum* (Lövenklev et al., 2004). Similarly, nutrient sources such as glucose, casein and glycine enhanced toxin production in *C. botulinum* type A, *C. botulinum* Hall A and Okra B, and *C. botulinum* serotype F respectively whereas tryptophan and arginine, proline or glutamate reduced toxin production in *C. botulinum* type E and C. *botulinum* Hall A and Okra B respectively (BONVENTRE & KEMPE, 1960; Leyer & Johnson, 1990; Licona-Cassani et al., 2016). The higher BoNT production in CO_2 rich media is possibly linked to bicarbonate formation (Lövenklev et al., 2004). Several two component systems such as CLC_0661/CLC_0663 (a homologue to phosphate sensing TCS PhoP/PhoR) and quorum sensing mechanisms (*agr-2*) have been shown to regulate BoNT synthesis (Connan et al., 2012; Cooksley et al., 2010). Further, BoNT is suggested to aid in the bacterial propagation and nutrient acquisition in the environment (Benoit, 2018). Host susceptibility to BoNT toxin may also be an important determinant in life cycle dynamics (Dohms & Cloud, 1982; Wobeser, 1988). In a carcass-necrophagous flies life cycle, maggots feeding on carcasses carry clostridia and its BoNT, which is then transmitted to other host feeding on the maggot, where BoNT kills the host and further facilitates its propagation (Benoit, 2018). Hence, the primary purpose of these toxins is to facilitate survival and spread of the bacteria in the environment outside the human host. Abundance of *C. botulinum* spores in the environment are driven by local soil, sediment, and water conditions, and elevated temperatures in summer months can favor the growth of *C. botulinum* in carcasses or in decomposing organic matter (Vidal et al., 2013; White & Oliphant, 1982; Wobeser & Galmut, 1984; Wobeser, Marsden, & MacFarlane, 1987; Woo et al., 2010).

2.2.2.4 *Clostridium tetani*

2.2.2.4.1 Taxonomy, Distribution, and Disease

Clostridium tetani is an anaerobic spore forming rod that is the causative agent of tetanus. Tetanus is also known as 'lockjaw' disease and is characterized by severe muscle contractions particularly at neck and jaw regions disrupting an individual's speech, swallowing, and breathing ability (Centers for Disease Control and Prevention, 2019; World Health Organization, 2020d). The disease can cause almost 100% mortality if immediate treatments are not available (George;, Jesus;, & Vivekanandan, n.d.). The bacterium is found in soil and is transmitted to humans via exposure to deep wounds (George; et al., n.d.). Tetanus can be classified as localized, generalized, or cephalic based on the location of the disease (George; et al., n.d.). The disease is of concern in unvaccinated mothers and their newborns(World Health Organization, 2020d). Tetanus in newborns is classified as "neonatal tetanus" and is linked to unsafe birth practices mainly in developing nations (George; et al., n.d.; World Health Organization, 2020d). Generalized tetanus is characterized by muscle spasms, difficulty in swallowing and breathing activity, and tonic posture (Boushab, Fall-Malick, Savadogo, & Basco, 2018). Localized tetanus occurs at specific sites and can cause rigidity, contractions, and lower motor neuron dysfunction of the involved muscle (Boushab et al., 2018; Chaudhary, Karki, Bhatta, & Pradhan, 2013; George; et al., n.d.). Cephalic tetanus is a rare type of localized tetanus involving cranial nerves (Alhaji, Abdulhafiz, Atuanya, & Bukar, 2011), and often occurs as a complication of ear infections or head injuries (Alhaji et al., 2011; Boushab et al., 2018; George; et al., n.d.). Cephalic tetanus can cause trismus, neck stiffness, facial muscle spasms and cranial nerve paralysis (Alhaji et al., 2011).

2.2.2.4.2 Pathogenesis

The characteristic muscular contraction of tetanus is caused by tetanus neurotoxin (TeNT), which is also known as tetanospasmin (George; et al., n.d.). *C. tetani* also possess various virulence factors such as collagenase, surface attached proteins and tetanolysin toxin that contributes to wound colonization and disease pathology (Chapeton-Montes et al., 2019). Tetanolysin is a pore forming toxin similar to botulinolysin and pneumolysin of *C. botulinum* and *Streptococcus pneumoniae*, respectively (Los, Randis, Aroian, & Ratner, 2013). It can lyse macrophages and platelets (Chapeton-Montes et al., 2019; Cox, Hardegree, & Fornwald, 1974), is cardiotoxic and is suggested to cause complications related to cardiovascular systems in tetanus (Cox et al., 1974; Los et al., 2013). However, major symptoms of tetanus are caused by tetanospasmin (Los et al., 2013). Tetanospasmin is considered the most toxic substance known after botulinum toxin (Masuyer, Conrad, & Stenmark, 2017). Tetanospasmin and botulinum toxins have structural and functional similarities and are suggested to have evolved from a common ancestor (Chapeton-Montes et al., 2019; Masuyer et al., 2017). However, these toxins differ in their disease pathology (Masuyer et al., 2017). Tetanospasmin blocks γ-aminobutyric acid (GABA) and glycine release in the nervous system to cause characteristic muscular contraction of tetanus whereas botulinum toxin blocks acetylcholine release to cause flaccid paralysis (George; et al., n.d.; Masuyer et al., 2017). It is suggested that tetanolysin facilitate dissemination of tetanospasmins inside the host (Ferrarotti, 2017).

2.2.2.4.3 Toxin Production in the Environmental Context

Tetanospasmin production is enhanced in the presence of nutrients such as casein and reduced in the presence of nutrients such as arginine, proline, or glutamate (Licona-Cassani et al., 2016). An exact function of tetanus neurotoxin in the environment is not known. However, it

is suggested that the rapid killing of hosts by the toxin could protect *C. tetani* against host immune systems and allow formation of spores for further spread (Rudkin, McLoughlin, Preston, & Massey, 2017).

2.2.3 Both aquatic and terrestrial habitats

2.2.3.1 *Nocardia*

2.2.3.1.1 Taxonomy, Distribution, and Disease

Nocardia are gram positive, weakly acid-fast, aerobic, pleomorphic actinomycetes (Pujic, Beaman, Ravalison, Boiron, & Rodríguez-Nava, 2015). Members of *Nocardia* are found worldwide in soil, water, and foliage. Bacterial cells can be disseminated through the air and inhaled to cause pulmonary nocardiosis; and puncture wounds can introduce bacteria to subcutaneous tissue and the blood stream (Rawat & Sharma, 2019). Infection with *Nocardia* can also impact the central nervous system leading to lesions and abscesses in the brain. Infection with *Nocardia* spp. can be difficult to diagnose due to similar presentation as other diseases and slow growth rate, and the nonspecific symptoms can vary from mild to severe. *Nocardia* spp. are opportunistic pathogens; they pose the greatest risk to those who are immunocompromised, such as patients following transplant, those with AIDS or other chronic diseases, and those who have a bacterial or viral co-infection (Dawar, 2016). Nocardiosis is caused by a variety of species in the genus; the most widespread species found in the United States is *Nocardia asteroides sensu stricto* type VI, while *Nocardia brasiliensis* is more commonly observed in humid, tropical areas (Saubolle & Sussland, 2003).

2.2.3.1.2 Pathogenesis

Nocardia's virulence is increased through inhibition of phagosome-lysosome fusion, the production of superoxide dismutase and catalase, and the formation of persister cells (Errington, Mickiewicz, Kawai, & Wu, 2016; Gonzalez-Carrillo et al., 2016). The secreted macrocyclic toxin, HS-6, identified in *N. otitidiscaviarum*, also causes significant damage to host pancreas, liver, stomach, small intestine, thymus, and heart, though its role in pathogenesis has been questioned (Beaman & Beaman, 1994).

2.2.3.1.3 Toxin Production in the Environmental Context

HS-6 has antifungal activity and could play role in outcompeting other microbes in the environment and during infection (Pujic et al., 2015). Genes encoding putative virulence factors, such as cord factor, vibriolysin and other toxin proteins, have since been detected in the genomes of several analyzed *Nocardia* strains, some of which were environmental isolates, though the function of these toxin in the environment remains unknown (Han et al., 2019; Vera-Cabrera, Ortiz-Lopez, Elizondo-Gonzalez, & Ocampo-Candiani, 2013; Yasuike et al., 2017).

2.2.3.2 *Burkholderia pseudomallei*

Burkholderia pseudomallei is one of more than one-hundred species of obligate aerobic, non-spore-forming, gram negative rods within the family Burkholderiaceae, found ubiquitously as a soil saprophyte, in surface water, and in some species of free-living amoeba ("Genus: Burkholderia," n.d.; Kaestli et al., 2015; Noinarin, Chareonsudjai, Wangsomnuk, Wongratanacheewin, & Chareonsudjai, 2016). *B. pseudomallei* is the causative agent of Melioidosis, a disease resulting in high mortality in humans and pigs, goats and sheep. It occurs less often in cattle, horses, dogs, rodents, birds, dolphins, tropical fish, primates in wild and

laboratory animals (Agriculture, n.d.). The disease is endemic in Asia and Northern Australia (Perumal Samy, Stiles, Sethi, & Lim, 2017). Infection occurs through percutaneous inoculation, inhalation, aspiration, and ingestion, that can present as asymptomatic infection, localized skin ulcers, abscesses, chronic pneumonia mimicking tuberculosis, and fulminant septic shock (Perumal Samy et al., 2017).

2.2.3.2.1 Pathogenesis

Though many of the *B. pseudomallei* virulence factors are uncharacterized, toxic effector molecules have been identified that include a protease resistant cytolethal toxin (CLT) that inhibits DNA and protein synthesis (HAASE, JANZEN, BARRETT, & CURRIE, 1997; Samy et al., 2017) and cell elongating toxin, a protease sensitive and heat labile toxin promoting cell elongation and growth arrest (HAASE et al., 1997). *Burkholderia* Lethal Factor 1 (BLF1) is another major toxin that inhibits translation and has cytotoxic activity against eukaryotic cells (Samy et al., 2017). Additionally, *B. pseudomallei* secretes a cytotoxic exolipid with activity against phagocytic (HL60) and nonphagocytic (HeLa) cell lines (Häußler, Nimtz, Domke, Wray, & Steinmetz, 1998). And finally, a toxic polyketide-peptide hybrid bactobolin was identified that also exploits host translation machinery (R. R. Wong, Kong, Lee, & Nathan, 2016).

2.2.3.2.2 Toxin Production in the Environmental Context

Burkholderia pseudomallei can persist for long periods under low-nutrient conditions; the organism has been cultured from distilled water 16 years after inoculation (Pumpuang et al., 2011). *Burkholderia pseudomallei* grows best in soil with 15% water content, and most infections occur during the rainy season (Y. S. Chen, Chen, Kao, & Chen, 2003; Duangurai, Indrawattana, & Pumirat, 2018; A. J. Merritt & Inglis, 2017). An investigation by Ulrich, et al

(2004) demonstrated that *B. pseudomallei* encodes multiple luxIR genes, and infection with quorum sensing mutants reduces animal pathogenicity, but does not affect exotoxin secretion, suggesting a role in environmental adaptation (Ulrich et al., 2004). Further, CLT activity varies between isolation source, with clinical isolates from severe disease cases showing higher cytotoxicity than less severe disease presentations and environmental soil isolates, with no difference in activity of cell elongation toxin between isolates (HAASE et al., 1997). Both environmental and laboratory assays suggest differential levels of toxin expression when exposed to various environmental conditions. The authors speculated that toxin production may be induced in the presence of liquid (i.e. in growth medium, or in the soil after heavy rainfalls), but suppressed under dry conditions, or by other mechanisms (HAASE et al., 1997) though this hypothesis remains understudied. Additionally, regulation and functioning of bactobolin and the cytotoxic exolipid within natural environments is unknown.

2.2.3.3 *Chromobacterium violaceum*

Chromobacterium violaceum are free- living, gram negative Betaproteobacteria, occupying soil and freshwater of global tropical and subtropical regions (Ciprandi et al., 2013). *Chromobacterium* contains nine species, but only *C. violaceum* and *C. haemolyticum* have been associated with human illness (Batista & da Silva Neto, 2017). *C. violaceum* is an environmental pathogen that can infect both animals and humans and rapidly progresses to septicemia. Transmission occurs through ingestion of contaminated seafood or water and through cutaneous trauma, with high mortality rates, ranging from 53-80% (Justo & Durán, 2017). As a free-living bacterium, adaptability in harsh environments outside organismal habitation is critical to its survival.

2.2.3.3.1 Pathogenesis

Investigations into the *C. violaceum* genome identified several putative virulence factors, including type II and type III secretion systems, cytolytic toxins (hemolysins and leukotoxins), metalloproteases, and lipases (Alves De Brito et al., 2004). Mass spectrometry confirmed genome findings by identifying proteins in the culture supernatant, including those with hemolytic activity, collagenase and a putative porin (Castro-Gomes et al., 2014).

2.2.3.3.2 Toxin Production in the Environmental Context

The bacterium has large numbers of genes devoted to stress adaptations and uses quorum sensing to regulate a number of inducible systems (de Vasconcelos et al., 2003). Other notable features of *C. violaceum* that impact the bacteria's surroundings are the metabolites they produce: Violacein and hydrogen cyanide. Violacein is a purple pigmented secondary metabolite secreted from the bacterium which exhibits antimicrobial activity (Lichstein & Van De Sand, 1945; Rettori & Durán, 1998). Its known functions are in quorum sensing, for eliminating microbial and eukaryotic competitors including *M. tuberculosis, Leishmania*, *Trypanosoma cruzi* and *Plasmodium* (Durán et al., 2016; Leon, Miranda, De Souza, & Durán, 2001; Lopes et al., 2009; McClean et al., 1997), and protection against UV radiation (de Vasconcelos et al., 2003; Durán et al., 2016). Furthermore, *C. violaceum* secretes Hydrogen cyanide (HCN), a corrosive chemical that impacts accumulating biofilms on surfaces by eroding metals and minerals (Carepo et al., 2004; Fairbrother et al., 2009; Michaels & Corpe, 1965). Production of HCN may also act as a defense mechanism against predation by grazing aquatic macroinvertebrates. HCN produced by *C. violaceum* increases larval mosquito mortality, and has led to the investigation of *C. violaceum* as a potential biocontrol agent (Short, Van Tol, MacLeod, & Dimopoulos, 2018). Hemolytic activity was also detected in a clinical isolate and in a soil isolate, with no difference

in activity between the two (D. P. Miller, Blevins, Steele, & Stowers, 1988). And, a subsequent proteomics study using a strain isolated from freshwater identified a hemolysin with 40 % sequence identity to *V. parahaemolyticus* and *L. pneumophila* cytolytic toxins (Ciprandi et al., 2013). Ecological roles of these toxins have not been identified, though one could speculate that activity could be against macroinvertebrates, owing to *C. violaceum's* mosquitocidal activity (Short, Van Tol, MacLeod, et al., 2018; Short, Van Tol, Smith, Dong, & Dimopoulos, 2018).

2.2.4 Toxin Producing Eukaryotic Environmental Pathogens

Toxic secondary metabolites produced by fungi range from antibiotics to carcinogenic compounds (J. W. Bennett & Klich, 2003). These mycotoxins can contaminate crops and cause human diseases ranging from food poisoning to hepatocellular carcinoma, thereby imposing a significant health and economic impact (J. W. Bennett & Klich, 2003; Zain, 2011). Aflatoxins produced by *Aspergillus* spp remain the most potent out of more than 100 mycotoxins identified to date (World Health Organization, 2018b). Clinically important myctotoxins include aflatoxins, citrinin, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone that are produced by *Aspergillus, Penicillium, Claviceps, Fusarium* and *Alternaria* (J. W. Bennett & Klich, 2003; Steyn, 1995). In humans, mycotoxins can cause digestive illnesses, malnutrition, tissue necrosis, neural tube defects, nephrotoxicity, cancer and even death (Zain, 2011). Mycotoxin exposure is usually due to accidental ingestion, inhalation or inoculation of the toxin, and toxin production does not usually confer a fungal growth advantage within a vertebrate hosts (except gliotoxin that can suppress host immune systems) (J. W. Bennett & Klich, 2003). As with prokaryotic toxins, mycotoxins may be important in natural fungal environments for surviving predation by amoeba, slime molds, soil arthropods and nematodes, and may be involved in interdomain interactions, in quorum sensing, and in biofilm formation (Venkatesh &

Keller, 2019). The selection of these toxin producing fungi thus allows survival in the natural soil and aquatic environments, with some being able to establish a productive infection within invertebrate, vertebrate, and plant hosts.

2.2.4.1 *Aspergillus* spp.

2.2.4.1.1 Taxonomy, Distribution, and Disease

Aspergillus are filamentous fungi that can reside in a wide variety of environmental habitats such as air, soil and detritus (Mousavi, Hedayati, Hedayati, Ilkit, & Syedmousavi, 2016). *Aspergillus* consists of approximately 344 species; however, only a few of them such as *A. fumigatus*, *A. flavus*, *A. terrus*, *A. niger* and *A. nidulans* are pathogenic to humans, mostly in immunocompromised individuals (Tsang, Tang, Lau, & Woo, 2018; Varga, Baranyi, Chandrasekaran, Vágvölgyi, & Kocsubé, 2015). *Aspergillus* transmission occurs through inhalation of spores aerosolization of contaminated water (Warris & Verweij, 2005). The diseases caused by *Aspergillus* spp are collectively termed as 'aspergillosis' and can range from allergy to chronic and invasive aspergillosis involving multiple organ systems (Centers for Disease Control and Prevention, n.d.). Allergic bronchopulmonary aspergillosis (ABPA) affects over one million people worldwide, whereas invasive aspergillosis is of relatively low prevalence, mainly affecting immunocompromised individuals though still caused around 15, 000 hospitalizations in 2014, ranking 4^{th} as the leading cause of death in intensive care units (Centers for Disease Control and Prevention, n.d.). Mycotoxins, specific enzymes, pigments and thermotolerance are virulence factors aiding survival in natural environments and within hosts (Mousavi et al., 2016).

2.2.4.1.2 Pathogenesis

Aspergillus mycotoxicosis is mainly caused by ingestion of pre-formed toxins such as aflatoxins, sterigmatocystin, ochratoxins and fumonisins in food products, resulting in toxic and carcinogenic effects within the liver, esophagus and kidneys (Varga et al., 2015). Besides direct toxicity, mycotoxins can damage epithelial cells (e.g. verruculogen) and suppress immune functioning (e.g. gliotoxin, aflatoxin and T2 toxin) to aid in *Aspergillus* colonization and survival (Mousavi et al., 2016).

2.2.4.1.3 Toxin Production in the Environmental Context

Aflatoxins are polyketide mycotoxins produced by *Aspergillus* section *Flavi,* mainly by *A. flavus* (aflatoxins B1 and B2) and *A. parasiticus* (aflatoxins B1, B2, G1 and G2) (Abdel-Hadi, Schmidt-Heydt, Parra, Geisen, & Magan, 2012; Calvo, Bok, Brooks, & Keller, 2004). In *A. falvus,* aflatoxin production is mediated by a 90 kb genomic DNA locus with multiple genes involved in a multi-step biosynthetic pathway to convert acetate to aflatoxin (Prieto, Yousibova, & Woloshuk, 1996; Yu et al., 1995). Aflatoxin production depends on several environmental factors and is increased at 29-30˚C, pH 3.4-5.5, higher water activity and in the presence of simple sugars and nitrogen in the form of ammonium. But nitrate, basic pH, temperature below 25˚C and above 37˚C and some plant volatiles suppresses toxin synthesis (Bhatnagar, Cary, Ehrlich, Yu, & Cleveland, 2006). In a model described by Abdel-Hadi *et al.* (2012), temperature and water activity affected aflatoxin production by lowering transcription of *aflR,* a key regulatory gene, and *aflD* and *aflM,* genes involved in early steps in aflatoxin production (Abdel-Hadi et al., 2012). Further, higher aflatoxin production has been observed in *A. flavus* in response to oxidative stress suggesting a possible role as a fungal antioxidant in the environment (Fountain et al., 2014). Moore *et al.* (2017) discussed that non-aflatoxigenic strains of *A. flavus*

can become toxigenic through genetic recombination and also that aflatoxigenic strains have a higher ability to adapt to several abiotic factors, including soil type, precipitation, and temperature, suggesting aflatoxin provides a fitness advantage in natural environments and under changing climates (Moore et al., 2017). Janzen suggested that aflatoxin production is favored in the presence of some microbes, birds, mammals or insects with which the competing fungus produces the compound as a means of chemical warfare to reduce attractiveness of the resource (Janzen, 1977). And when competitors are absent, the cost of toxin production favors non-producers, driving genetic variation. Finally, balancing selection of genetic variation for aflatoxin production in *A. flavus* was shown to be driven by interference competition as protection from insect fungivory (Drott, Lazzaro, Brown, Carbone, & Milgroom, 2017).

2.2.4.2 *Fusarium* spp

2.2.4.2.1 Taxonomy, Distribution, and Disease

Fusarium spp are filamentous fungi that inhabit soil, plant material and water (Nucci & Anaissie, 2007). There are over 100 *Fusarium* species identified, among which *F. solani, F. oxysporum, F. verticillioidis* and *F. moniliforme* are the most common human pathogens (Al-Hatmi, Hoog, & Meis, 2019; Nucci & Anaissie, 2007). *Fusarium* exhibits inter-kingdom pathogenicity and can infect both plants and humans (Al-Hatmi et al., 2019). In plants, it is one of the most destructive fungal pathogens (Al-Hatmi et al., 2019). In humans, *Fusarium* causes a wide range of disease presentations such as keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, fungemia, endophtalmitis, septic arthritis and osteomyelitis (Nucci & Anaissie, 2007). Transmission occurs through airborne or cutaneous routes (Nucci & Anaissie, 2007; Nucci et al., 2013), and infections are mainly localized, though disseminated and fatal infections

can occur in immunocompromised patients (Al-Hatmi et al., 2019; Dehal & Quimby, 2019; Nucci & Anaissie, 2007).

2.2.4.2.2 Pathogenesis

Several virulence factors including mycotoxins, proteases, collagenases, mitogen-activated protein kinase, a pH response transcription factor, and a class V chitin synthase, aid in pathogenicity to promote adherence, dissemination and immune suppression (Nucci & Anaissie, 2007). Major mycotoxins produced by *Fusarium* include fumonisins, trichothecenes and zearalenone that are found in and on corn, barley, rye, safflower seeds, wheat, and mixed feeds (J. W. Bennett & Klich, 2003). Other *Fusarium* toxins include fusarins, fusaric acid, moniliformin, enniatins and beauvericins (Perincherry, Lalak-KaNczugowska, & St˛epie, 2019). These toxins can cause plant rot, and are also implicated in various human diseases such as esophageal cancer (fumonisins and fusarins), neural tube defects (fumonisins), immunological problems, dermatitis, gastroenteritis and alimentary toxic aleukia (Trichothecenes) and hepato- and nephrotoxicity (Zearalenone) (J. W. Bennett & Klich, 2003; Jimenez-Garcia et al., 2018; Perincherry et al., 2019). Four trichothecenes, trichothecene A (T2, HT2 and diacetoxyscirpenol), B (deoxynivalenol and nivalenol), C and D (Jimenez-Garcia et al., 2018) inhibit DNA, RNA and protein synthesis in eukaryotes (J. W. Bennett & Klich, 2003; Jimenez-Garcia et al., 2018). Trichothecene B (deoxynivalenol) aids in disseminating *Fusarium* from plants by preventing cell-wall thickening (Perincherry et al., 2019). In *F. graminearum*, deoxynivalenol also prevents programmed cell-death in host *Arabidospis thaliana* to enhance physical and pathogenic interactions with *Burkholderia glumae* bacteria during polymicrobial infection of rice (Venkatesh & Keller, 2019). Deoxynivalenol and T2 and HT2 are immunosuppressive and increases risk of infection by other pathogens (Antonissen et al., 2014;

J. W. Bennett & Klich, 2003). *Fusarium* mycotoxins such as zearalenone, fumonisins, and deoxynivalenol also increase membrane permeability and affect host immunity to enhance diseases caused by other pathogens such as *Eimeria, Salmonella, E. coli, C. perfringens, Aspergillus spp* and *reovirus* (Antonissen et al., 2014). Furthermore, noncytotoxic concentrations of deoxynivalenol promoted intestinal invasion by *Salmonella* in pigs, and necrotic enteritis in broiler chickens through intestinal leakage and increased nutrient availability to *C. perfringens* (Antonissen et al., 2014).

2.2.4.2.3 Toxin Production in the Environmental Context

Various biotic and abiotic factors such as microbial interaction, plant extract, nutrient availability, temperature, pH, oxidative stress and water activity influence toxin production (Perincherry et al., 2019; Venkatesh & Keller, 2019). In the environment, beauvericin, fusaric acid and deoxynivalenol safeguard fungi against other microbes by preventing invasion by bacteria such as *Ralstonia* and providing protection against various antimicrobials such as 2,4-diacetylphloroglucinol, and enzymes such as chitinase produced by bacteria and parasites (Venkatesh & Keller, 2019). Additionally, these toxins sequesters iron (fusaric acid), exhibit quorum quenching activity against bacteria (fusaric acid, zearalenone and fumonisin) and reduce biofilm formation in *Candida* (zearalenone) to enhance *Fusarium* competition and survival in natural environments (Venkatesh & Keller, 2019).

2.2.4.3 *Penicillium* spp

2.2.4.3.1 Taxonomy, Distribution, and Disease

Penicillium spp are economically and historically important filamentous fungi that are distributed worldwide in a diverse range of habitats such as soil, vegetation, air and food

products (Guevara-Suarez et al., 2016; Visagie et al., 2014). The genus is credited for being the producer of the first widely produced antibiotic, penicillin ("Discovery and Development of Penicillin," n.d.). There are 354 *Penicillium* species, some of which are medically, industrially and clinically important (Guevara-Suarez et al., 2016; Tsang et al., 2018; Visagie et al., 2014). For instance, *Penicillium* spp such as *P. citrinum, P. chrysogenum* and P. *decumbens* can cause superficial, invasive and disseminated infections in humans including keratitis, cutaneous infections, allergies, sick building syndrome, pneumonia, and fungal ball (Guevara-Suarez et al., 2016; Schwab & Straus, 2004). Other *Penicillium* spp such as *P. brevicompactum*, *P. purpurogenum,* and *P. canis* are implicated in infection in dogs (Guevara-Suarez et al., 2016). Though *P. chrysogenum* (formerly known as *P. notatum*) is implicated in human disease, it also produces penicillin and is used in the industrial production of several beta-lactam antibiotics (Bajaj et al., 2014). The genus also includes economically important species such as *P. roqueforti* and *P. nalgiovense* that are involved in the production of cheese and sausages, respectively (Tsang et al., 2018). In contrast, species such as *P. citreonigrum* spoil food and destroys crops to cause significant loss to food industries (Tsang et al., 2018). And a related genus, *Talaromyces marneffei* (formerly known as *P. marneffei*) causes fatal systemic infection in HIV-positive and other immunocompromised patients (Guevara-Suarez et al., 2016; Tsang et al., 2018).

2.2.4.3.2 Pathogenesis

Most *Penicillium* are considered saprophytes and contaminants, though some can cause disease in immunocompromised individuals, where invasive pulmonary infection has been reported (Ramírez, Hidrón, & Cardona, 2018). Citrinin is also produced by several species of

Penicillium that acts as a potent nephrotoxin in all animal species tested, and may act synergistically with another nephrotoxin, ochratoxin (JH, 2015).

2.2.4.3.3 Toxin Production in the Environmental Context

The functioning of penicillin as a bacteriocidal compound has already been discussed at the beginning of this work. But, other toxic secondary metabolites such as ochratoxins, patulin, citrinin, and penicillic acid are produced by a wide range of *Penicillium* and *Aspergillus* species (Frisvad, 2018; Greeff-Laubscher, Beukes, Marais, & Jacobs, 2019; JH, 2015). These toxins are contaminants of agricultural and food products such as cereals, rice, nuts, fruit juices and spices and cause mycotoxicosis upon ingestion of contaminated foods (Frisvad, 2018; Greeff-Laubscher et al., 2019; JH, 2015; World Health Organization, 2020c). But though toxic under some contexts, these secondary metabolites exhibit antitumor (citrinin), antibacterial (patulin, penicillic acid and citrinins), antifungal (patulin and citrinins) and antiviral (patulin) activities in other contexts (Frisvad, 2018; JH, 2015). Patulin and penicillic acid inhibits quorum sensing by targeting RhlR and LasR bacterial proteins (Rasmussen et al., 2005). This quorum sensing inhibition regulated *P. aeruginosa* virulence gene expression and enhanced biofilm susceptibility to tobramycin (Frisvad, 2018; Rasmussen et al., 2005). Ochratoxin production is also influenced by biotic and abiotic factors including the presence of competing microbes, moisture content and temperature (J. W. Bennett & Klich, 2003), and also functions to maintain chloride homeostasis to producing fungi in high salt environments (Hymery et al., 2014).

2.3 Concluding Remarks

Environmental pathogens have a community lifestyle and are often associated within or on host organisms such as invertebrates, plants, amphibians and reptiles-all of which are hotspots

for genetic exchange, emergence of pathogens, and are also often the intersection of human contact. These environmental microbes are adapted and evolutionarily equipped with traits and compounds to overcome innate defenses within the organismal and biofilm environments, that become useful, in some cases, for adaptation to humans, and also for the spread of novel virulence factors into existing mutualists or pathogens. Hence, environmental hotspots of emergence and preadaptation act as melting pots for the acquisition of novel genes, or for the loss of functions of genes, involved in host-microbe interactions that might enhance pathogenicity and virulence.

Therefore, considering relationships between microbe and host as only those leading to negative outcomes (i.e. disease) could be considered as myopic. In the same vein, considering environment as an existent presence merely surrounding humans leads to conceptual error in medical microbiology and disease ecology. Reducing environment simply to soil, water, and air outside of humans fails to account for macro- and micro- biotic and abiotic conditions within these locations. A large body of literature, including discussed above, has documented that biotic interactions can affect species response to abiotic environmental changes differently along environmental gradients, and that abiotic environmental changes can likewise influence the nature of biotic interactions (Klanderud, 2005; Kraft et al., 2015; C. K. Lee et al., 2019; Soininen, 2010). This underscores our understanding that the production of secondary metabolites as toxic compounds is context dependent and is shaped by evolutionary trade-offs at multiple scales (genetic, cellular, population), where production may provide fitness and environmental lifestyle advantages for the producing microbes that are unrelated to host interaction and disease.

But, because the emergence of infectious diseases involves many factors including microbial community interactions with the pathogen, the hosts and their environment, there is a developing need for interdisciplinary approaches to studying emerging infections and the role of these secondary compounds within natural environments to better understand the ecological dynamics, potential severity of modern infectious diseases, and for developing novel therapeutics (Ezenwa et al., 2015). This review, while highlighting some suggested and known reasons for toxin production, has revealed that there are a lot of unknowns remaining with regards to factors leading to production, and mechanisms of toxins in functioning in community inter- and intra-species interactions across a range of ecosystems and environmental conditions.

Assembly in host- and environment-associated microbial communities is a recent area of interest (Maignien, DeForce, Chafee, Eren, & Simmons, 2014; Nemergut et al., 2013). Testing assembly hypotheses in host-associated microbial communities, however, has been challenging for several reasons that include the following: first, the wide dispersal of microbes outside of a host makes characterization of regional pools difficult; second, the tools and disciplines of ecological theory are not part of the contemporary mindset in microbiology and infectious disease medicine, in general. Indeed, the accumulation of data in microbiology and medical science over the years requires the application of ecological, quantitative theory to provide organization, structure and mechanistic insights (Prosser et al., 2007). With this, community ecology concepts, along with modern genomic tools will aid to identify mechanisms of microbial molecular evolution that result in the persistence and dispersal of pathogens.

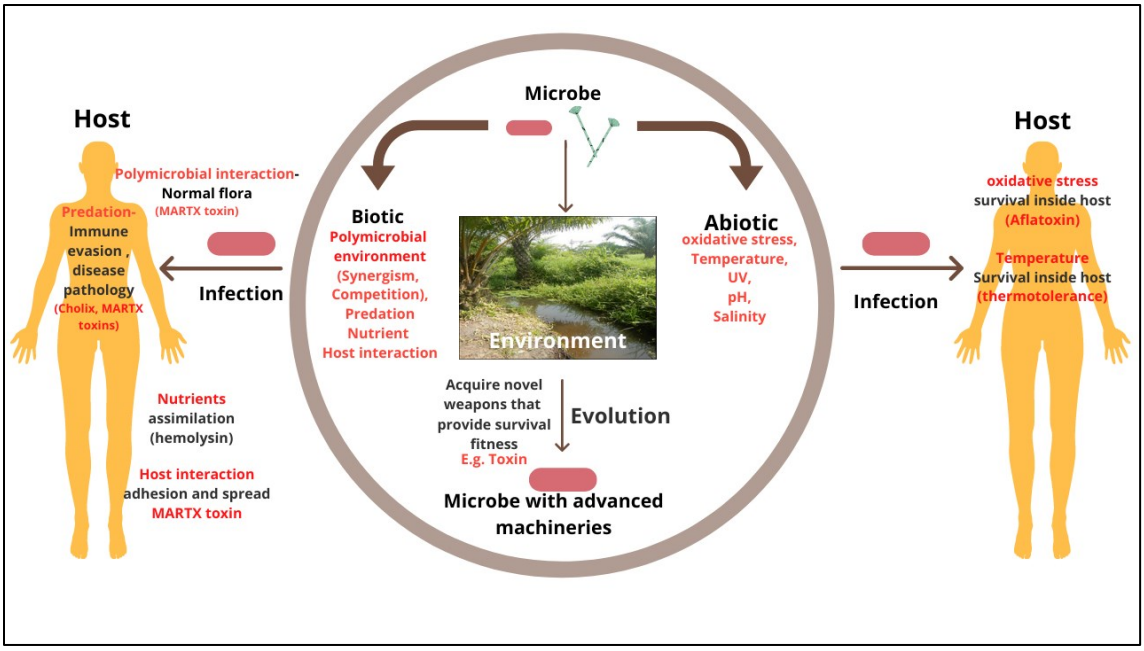

Figure 2.1 Coincidental evolution of virulence hypothesis.

According to the coincidental evolution hypothesis, microbes acquire traits to counteract with the abiotic and biotic challenges in their natural habitat that can act as virulence upon accidental infection to human host. These novel weapons such as toxin can be produced as a stress response to several biotic and abiotic factors such as microbial metabolites, oxygen concentration, temperature variation, pH and salinity. They can provide survival fitness by aiding nutrient assimilation, establishing symbiotic relationship with the host, enhancing competition, and protecting against predation during normal life cycle in the environment (indicated within the circle). However, these traits can confer virulence upon accidental infection of the susceptible host that occur outside of their normal life cycle (indicated outside of the circle). As shown in the figure, microbes acquire traits to compete in polymicrobial environment, protect itself against predation assimilate nutrients, interact with host, and cope with oxidative and heat stress. However, the machineries acquired enhance their survival inside the human host by facilitating nutrient acquisition (e.g. hemolysins) and protecting against oxidative (e.g. aflatoxin) and temperature stress (e.g. thermotolerance). They also provide competitive advantage against normal flora (MARTX toxin) and aid in adhesion spread, and immune evasion (e.g. MARTX toxin) and to cause disease pathology (e.g. cholix toxin) to provide virulence.

Table 2.1 Toxin producing environmental pathogens and role of their toxins in virulence and in environmental context

Toxin Producing Environmental Pathogen	Toxin produced	Toxin's role in virulence	Toxin production in environmental context
Vibrio cholerae	cholera toxin, cholix toxin, hemolytic exotoxin called Cytolysin (VCC), and Multifunctional autoprocessing repeats-in-toxin (MARTXvc) (Matson et al., 2007; Ou et al., 2009; Sakib et al., 2018)	cholera toxin (CT)- adhesion to intestinal epithelial cells and cyclic AMP release, cholix toxin - damage cellular function, hemolytic exotoxin called Cytolysin (VCC)- induce inflammatory activity and enhance colonization, (MARTXvc)- facilitate immune evasion (Matson et al., 2007; Ou et al., 2009; Sakib et al., 2018)	sensing nutrient concentrations, promoting adherence and motility, colonization and establishment of symbiotic relationships to copepods and chironomids, and other natural reservoirs, confer cytotoxicity and bacteriocin-like activity to protect the producing bacteria against predators and provide a competitive advantage against incompatible *Vibrio* strains and other microbes (Sakib et al., 2018; Unterweger et al., 2014)
Vibrio vulnificus	$MARTX_{Vv}$, hemolysin (Vvha), and metalloprotease (VvPE)	$MARTX_{Vv-}$ colonization, spread, immune evasion, and pathology (pore formation), Vvha- pathology (pore formation) (Hor & Chen, 2013) (Lo et al., 2011)	promote environmental fitness through biofilm formation on chitin, adherence to aquatic flora, resist predation Phenotypic switch (M. Y. Kim et al., 2006; Klein et al., 2018; Rahman et al., 2008)
Legionella pnuemophila	homogentisic acid (HGA), Rtx family toxins	Rtx family ("repeats-in toxin") that is involved in adherence, cytotoxicity, and pore formation (D'Auria et al., 2008)	Competitive advantage against other microbes (HGA), adherence, entry, intracellular survival and trafficking in amoebae, allowing persistence within its environmental reservoir (RTX toxin), growth and iron uptake under iron-limiting conditions (HGA) (Cirillo et al., 2002; T. C. Levin et al., 2019; Zheng et al., 2013)

Table 2.1 (continued)

Toxin Producing Environmental Pathogen	**Toxin produced**	**Toxin's role in virulence**	**Toxin production in environmental context**
Mycobacterium *ulcerans*	Mycolactone	Pathology (tissue necrosis), immunosuppression	attractant for mosquito blood-feeding, and an oviposition stimulant, postulated roles in quorum sensing or quenching mechanism, and in stress response (Chakraborty & Kumar, 2019; Sanders et al., 2017; Sanhueza et al., 2019)
Bacillus cereus	Cereulide, enterotoxin (non-hemolytic enterotoxin (Nhe), and the hemolysin BL (HBL)), hemolysins I- IV (Ramarao & Sanchis, 2013)	Cereulide-Disease pathology (inhibit mictochondrial activity-leads to liver failure), Enterotoxin- disease pathology (forms pore to cause diarrhea) Hemolysin- disease pathology (pore formation), Spread, nutrient acquisition (Ehling-Schulz et al., 2015) (Gohar et al., 2008) (Ramarao & Sanchis, 2013) (Sastalla et al., 2013)	Competitive advantage against other microbes, nutrient acquisition, and adaptation to specific niche (cereulide) (Ehling-Schulz et al., 2015) (Gohar et al., 2008)
Bacillus anthracis	anthrax toxin	Disease pathology (edema and cell destruction), dissemination and persistence in a host, immune evasion (Turnbull, 1996) (Loving et al., 2009))	Dispersal and transmission to hosts, proliferate and maintain its existence (Fasanella, 2013; Ganz et al., 2014)

Table 2.1 (continued)

Toxin Producing Environmental Pathogen	**Toxin produced**	**Toxin's role in virulence**	**Toxin production in environmental context**
Clostridium botulinum	α-Clostripain, BoNT, Hemolysin A and collagenase	posttranslational modification of neurotoxin (Alpha-clostripain), facilitates absorption of botulinum toxin and nutrient acquisition in the intestine (Botulinolysin (BLY)), Disease pathology- blocks release of acetylcholine, a principle neurotransmitter to cause descending flaccid paralysis (Botulinum) (T. Suzuki et al., 2016) (Abdela et al., 2016) (Nigam & Nigam, 2010) (World health organization, 2018)	support the bacterium's saprophytic life cycle by playing role in binding and degradation of tissues, bacterial propagation and nutrient acquisition in the environment (Benoit, 2018; Chellapandi & Prisilla, 2018; Doxey et al., 2008)
Clostridium tetani	Tetanospasmin and tetanolysin	Disease pathology (Tetanospasmin), Immune evasion (Tetanolysin), tetanus complications (Tetanolysin) and dissemination of Tetanospasmin (Tetanolysin) (Cox et al., 1974) (Los et al., 2013) (Ferrarotti, 2017))	Spread (Rudkin et al., 2017)
Nocardia	macrocyclic toxin (HS-6)	Disease pathology (attacking organs such as the liver, pancreas, and kidneys) (Beaman & Beaman, 1994)	Competitive advantage (Antifungal activity against Cryptococcus neoformans) (Pujic et al., 2015)

Table 2.1 (continued)

Toxin Producing Environmental Pathogen	**Toxin produced**	**Toxin's role in virulence**	**Toxin production in environmental context**
Burkholderia pseudomallei	cytolethal toxin, Cell elongating toxin, *Burkholderia* Lethal Factor 1 (BLF1), cytotoxic exolipid, toxic polyketide-peptide hybrid bactobolin (HAASE et al., 1997) (Häußler et al., 1998) (R.-R. Wong, Kong, Lee, & Nathan, 2016)	cytolethal toxin (inhibits DNA and protein synthesis), Cell elongating toxin (promote cell elongation and growth arrest), *Burkholderia* Lethal Factor 1 (BLF1) (inhibits translation, cytotoxicity), cytotoxic exolipid, bactobolin (HAASE et al., 1997)	Environmental adaptation (Ulrich et al., 2004)
Chromobacterium violaceum	hemolysins and leukotoxins (Alves De Brito et al., 2004)		could be against macroinvertebrates (*C. violaceum* has been shown to have mosquitocidal activity)
Aspergillus spp (Eukaryotic)	aflatoxins, sterigmatocystin, ochratoxins and fumonisins, verruculogen, gliotoxin, T2 toxin (Varga et al., 2015)	toxic and carcinogenic effects on various organs, damage the epithelium (e.g. verruculogen), suppresses immune system (e.g. gliotoxin, aflatoxin and T2 toxin) that facilitate colonization and survival of fungi inside human host (Mousavi et al., 2016)	Aflatoxin-antioxidant to cope with oxidative stress, fitness advantage in natural environments and under changing climates, interfere competition (Drott et al., 2017; Fountain et al., 2014; Moore et al., 2017)

Table 2.1 (continued)

Toxin Producing Environmental Pathogen	**Toxin produced**	**Toxin's role in virulence**	**Toxin production in environmental context**
Fusarium spp **(**Eukaryotic)	fumonisins, trichothecenes and zearalenone, fusarins, fusaric acid, moniliformin, enniatins and beauvericins (J. W. Bennett & Klich, 2003) (Perincherry et al., 2019)	Trichothecenes inhibit DNA, RNA and protein synthesis in eukaryotes, Deoxynivalenol aid in spread of *Fusarium* spp in plants by preventing cell-wall thickening, deoxynivalenol also prevents programmed cell-death in host *Arabidospis thaliana* to aid pathogenesis and enhance microbial interaction to bacteria *Burkholderia glumae* during polymicrobial infection of rice, Deoxynivalenol and toxin T-2 are immunosuppressive (J. W. Bennett & Klich, 2003) (Jimenez-Garcia et al., 2018) (Perincherry et al., 2019) (Venkatesh & Keller, 2019) (Antonissen et al., 2014)	Competitive advantage against other microbes (beauvericin, fusaric acid, deoxynivalenol and zearalenone), Iron sequestration (fusaric acid), quorum quenching activity against bacteria (fusaric acid, zearalenone and fumonisin) (Venkatesh & Keller, 2019)
Penicillium spp **(**Eukaryotic)	ochratoxins, patulin, citrinin, penicillic acid (Frisvad, 2018) (JH, 2015))	Citrinin, ochratoxin (nephrotoxin) (JH, 2015)	Competitive advantages against other microbes (antibacterial (patulin, penicillic acid and citrinins), antifungal (patulin and citrinins) and antiviral (patulin), quorum quenching (patulin and penicillic acid), Ochratoxin maintains chloride homeostasis to provide fitness advantage to producing fungi in high salt environment (Frisvad, 2018) (JH, 2015) (Hymery et al., 2014)

CHAPTER III

RESEARCH SIGNIFICANCE

Understanding the stress response of *M. ulcerans* and its impact on mycolactone and global gene regulation is significant as it provides insights on its reservoir and during transmission and subsequent pathogenesis. Organisms acquire new traits to survive and overcome adverse environmental conditions, thus playing a significant role in their evolution. Stresses such as high temperature, low oxygen and UV radiation that bacteria can encounter in their natural habitat or during infection to a host can regulate several stress response genes that can facilitate its survival and virulence.

BUD is associated with stagnant water (Campbell et al., 2015; Andrés Garchitorena et al., 2014; R. W. Merritt et al., 2010; Noeske et al., 2004). Similarly, studies have reported an asymptomatic colonization of the intestinal tract of mammals by *M. ulcerans* (O'Brien et al., 2014). Further, BUD established efficient disease pathology during intradermal injection of *M. ulcerans* but not when introduced through an abrasion site (Williamson et al., 2014). These associations of *M. ulcerans* and BUD in natural environments with diverse oxygen and temperature conditions raises the question on these abiotic effects on *M. ulcerans* survival and regulation during pathogenesis. In this collective set of studies, *M. ulcerans* was first exposed to dynamic oxygen and temperature, conditions that bacteria can encounter in their natural habitat and during infection to the host, to better understand its regulation of stress response and mycolactone expression, and to discern both its niche habitat and pathogenesis. Additionally,

other biotic and abiotic factors such as use or interfering with bacterial quorum sensing molecules, nutrient composition, sunlight, and salinity will also provide hints toward its natural niche environment. Hence, it is important to understand the effect of factors such as nutrient composition and UV radiation on *M. ulcerans* to provide insights on where *M. ulcerans* may be replicating, and what may be its natural reservoir.

Investigating the role of mycolactone during *M. ulcerans*-polymicrobial interaction is significant research because it aids to understand *M. ulcerans* ecology, which is one of the current critical barriers in understanding its reservoir and modes of transmission. During infection of a host, an environmental pathogen such as *M. ulcerans* encounters both normal host floras, some with opportunistic pathogen potential, which can lead to either mutualistic or antagonistic interactions. Thus, environmental pathogens often produce secondary metabolites as adaptive defenses or means of persistence within the polymicrobial host environment. It has been suggested that *M. ulcerans* acquired plasmid pMUM001 during emergence from its *M. marinum* progenitor. This, along with a reduced genome from *M. marinum* suggest a new niche environment, and suggests a specific role of mycolactone in adapting to this new environment (Yip et al., 2007).

The structure similarity of mycolactone to homoserine lactones and mycolactone's continual increase after bacterial cultivation reached a plateau in an animal model suggests it may be used in quorum sensing mechanism, and may thus influence microbial community structure or function (Fred Stephen Sarfo et al., 2013). It is also suggested that environmental pathogens acquire traits whose primary function is to provide a fitness advantage in natural host-free environments, however, can act as virulence factors upon infection to hosts (S. Sun, Noorian, & McDougald, 2018). For example, secondary metabolites such as mycotoxins

produced by various fungi has been shown to exhibit quorum quenching mechanisms (Frisvad, 2018; Venkatesh & Keller, 2019). Further, compounds that are toxins in human hosts have been shown to provide natural protection against predators to the producing microbe, as well as to neighboring microbes thereby driving microbial community structure in their local vicinity(Jousset, 2012). We therefore also conducted a study where we investigated the effect of mycolactone on microbial community structure in natural, aquatic habitats where *M. ulcerans* surveys had been found in high or in low abundance. The determination of how mycolactone impacts microbial community structure in natural, aquatic habitats could aid to understand quorum sensing and quenching ability of *M. ulcerans* and its impact on polymicrobial interactions. This could lead to means for prevention, treatment and policy.

Studies have also shown that Buruli ulcer wounds are colonized by several pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, however, without specific pathology associated with those pathogens (Kpeli et al., 2016). Further, quorum sensing molecules such as acyl homoserine lactone produced by *P. aeruginosa*, and signal peptides produced by other *Staphylococcus* species inhibit *S. aureus* quorum sensing mechanisms thereby exhibiting quorum quenching abilities (Qazi et al., 2006). In this study, long chain HSLs such as 3-oxo-C12-HSL bound and disrupted the *S. aureus* membrane, which also disrupted functions of other membrane associated proteins such as Agr-system proteins (Qazi et al., 2006). This quorum quenching ability is suggested to provide competitive advantage to *P. aeruginosa* against *S. aureus* during co-infection of wound or respiratory tract of cystic fibrosis patients (Qazi et al., 2006). These findings led us to investigate the chemical antagonism exhibited by mycolactone against the human skin organism, *S. aureus*. We showed that mycolactone suppresses *S. aureus* quorum sensing genes and phenotypes, suggesting its role in an inter-

bacterial interaction to provide competitive advantages against other bacteria with differing mechanisms for, or even higher virulence during infection to human hosts. Additionally, the Agr-system promotes dispersal of *S. aureus* biofilms (Archer et al., 2011). Hence, its downregulation may enhance biofilm formation which can protect *M. ulcerans* against external stresses. The finding is significant as it aids explaining polymicrobial interactions, and chemical defenses of environmental pathogen against other opportunistic pathogens during skin infection and disease establishment. Further, it suggests a functional role of mycolactone for *M. ulcerans* and provides preliminary data on possible candidacy of mycolactone as a treatment option against *S. aureus*. The acquisition of such knowledge is important in designing therapeutic strategies to treat BUD as a decrease in *M. ulcerans* and ultimately in mycolactone in these wounds could lead to enhanced virulence and pathogenicity by the remaining pathogens leading to delayed wound healing and paradoxical reactions (Yeboah-Manu et al., 2013). Additionally, determining mycolactone impact on quorum sensing molecules from other pathogens can have potential to be used as a therapeutic strategy. Overall, these studies provide insights to *M. ulcerans* in natural environments and during transmission and host infection, paving the way for means of prevention, early treatment, and policy.

CHAPTER IV

INVESTIGATING MYCOBACTERIUM ULCERANS STRESS RESPONSE TO UNDERSTAND ITS PATHOGENESIS AND ECOLOGICAL NICHE

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4.1 Abstract

Buruli ulcer disease (BUD) is a neglected tropical disease caused by an environmental pathogen *Mycobacterium ulcerans*. The major virulence factor of *M. ulcerans* is mycolactone, a lipid cytotoxin whose genes are encoded on the large plasmid pMUM001. Although an exact reservoir and mode(s) of transmission is unknown, the disease has been linked with slow-moving water with low oxygen concentration. Further, *M. ulcerans* have been shown to cause disease in guinea pigs following injection, but not when introduced to an open abrasion site, suggesting that puncture is necessary for efficient transmission and pathology. *M. ulcerans* has been suggested to be sensitive to UV due to termination in *crtI,* encoding a phytoene dehydrogenase, which is required for carotenoid pigment production. However, it is not clear whether *M. ulcerans* resides in a UV protected area within its natural environment, or has developed machineries, such as

mycolactone production, to protect itself, from sunlight, and other abiotic and biotic factors such as temperature, oxygen, or other microbes. Mycolactone, a polyketide derived macrolide, is structurally similar to N-acyl homo serine lactone, and mycolactone has been shown to increase continuously even after bacterial cultivation reached a plateau in an animal model, suggesting its function in quorum sensing mechanisms (Fred Stephen Sarfo et al., 2013). Despite this, the function and modulation of mycolactone in natural environments in response to dynamic abiotic and biotic conditions such as UV, temperature, oxygen, and other microbes has not been shown. In this study, we investigated modulation of global and mycolactone gene expression of *M. ulcerans* on exposure to various abiotic and biotic stresses to understand the reservoir, ecology, and pathogenesis of the bacterium. Mycolactone gene expression was downregulated on exposure to high temperature (37^0C) and did not change significantly on exposure to UV radiation, however, it was upregulated when exposed to microaerophilic conditions. Similarly, the combined stress of high temperature and low oxygen caused downregulation of mycolactone expression but upregulated several stress response genes. The upregulation of mycolactone expression under microaerophilic conditions suggests that mycolactone could provide a fitness advantage to *M. ulcerans* in a microaerophilic environment within its natural environment. Also, exposure of *M. ulcerans* to UV did not affect *M. ulcerans* growth and mycolactone expression. This suggests that *M. ulcerans* could have higher tolerance to UV exposure than previously thought and mycolactone may not provide fitness advantage against it. The stress response of *M. ulcerans* at high temperature (37^0C) and low oxygen (microaerophilic) condition was similar to *M. tuberculosis* response in hypoxic and intracellular environment. The upregulation of these stress response genes at expense of downregulation of mycolactone expression suggest energy

efficient strategy of *M. ulcerans* to modulate its gene expression to combat challenges in its natural habitat and during pathogenesis.

4.2 Introduction

Buruli ulcer disease (BUD) is a neglected tropical disease caused by an environmental pathogen *Mycobacterium ulcerans*. The disease is characterized by a painless nodule which can later develop into an ulcer (Williamson et al., 2014). It is the third most common mycobacterial infection and has been reported in over 33 countries worldwide (World Health Organization, 2016). BUD 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 (Beissner, Arens, et al., 2015; Hamzat & Boakye-Afram, 2011). The major virulence factor of *M. ulcerans* is mycolactone, a lipid cytotoxin whose genes are encoded on the large plasmid pMUM001 (T. P. Stinear et al., 2004). Mycolactone diffuses through healthy tissue leading to pathology further than the site of bacterial colonization (Sarfo et al., 2016). Mycolactone exposure impacts a wide variety of cells by modulating immune functioning, inhibiting immune regulating proteins, or by causing cytoskeletal rearrangement, cell cycle arrest, necrosis or apoptosis, depending on cell type (Sarfo et al., 2016).

Morbidity associated with BUD has, in part, been exacerbated by the fact that the mode of transmission of *M. ulcerans* remains unknown. Epidemiological evidence links incidence of BUD to slow moving aquatic habitats (Campbell et al., 2015; Merritt et al., 2010; Noeske et al., 2004). This has also been supported by the finding of *M. ulcerans* DNA in association with plant biofilms, water filtrand, soil, and invertebrates (Fyfe et al., 2007; Kotlowski et al., 2004; Vandelannoote et al., 2010; Williamson et al., 2012, 2008). Slow-moving water with low oxygen concentration has also been included as a risk factor for BUD (Campbell et al., 2015;

Andrés Garchitorena et al., 2014; Merritt et al., 2010; Noeske et al., 2004). Furthermore, *M. ulcerans* is an environmental pathogen that is assumed to be naturally residing in a complex community of aquatic life that is structured by biological interactions and abiotic environmental factors. And, the ability of *M. ulcerans* to cause disease in guinea pigs following injection, but not when introduced to an open abrasion site suggests that puncture is necessary to establish infection (Williamson et al., 2014). Results of those studies also suggest a possible role of higher temperature and lower oxygen within the puncture site compared to the abrasion site, in establishment of disease (Williamson et al., 2014). Low oxygen concentration and high temperature has been shown to upregulate virulence in environmental pathogens such as *Burkholderia cenocepacia* and *Vibrio vulnificus* (Phippen & Oliver, 2017; Sass et al., 2013). Additionally, temperature-regulated toxin production has been observed in pathogenic bacteria such as enterohemorrhagic *Escherichia coli* (EHEC), *Yersinia. enterocolitica* and *Bacillus anthracis* (Lam, Wheeler, & Tang, 2014). Therefore, understanding modulation of global and mycolactone 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 may also aid to understand virulence and pathogenesis of *M. ulcerans* leading to BUD.

It has been suggested that *M. ulcerans* is sensitive to UV due to termination in *crtL,* a gene responsible for carotenoid production (Zingue, Bouam, Tian, & Drancourt, 2018). As an exact reservoir of *M. ulcerans* is unknown, it is not clear whether *M. ulcerans* resides in areas within its natural environment that are protected from UV or has developed machineries to counteract adverse UV effects. But wild-type *M. ulcerans* produces bright yellow pigmented colonies, while mycolactone mutants are white, supporting that mycolactone may influence protection against UV through a pigment mediated mechanism.

M. ulcerans also shares a poly-microbial lifestyle in aquatic environments and in BUD wounds (Amissah et al., 2015). Many microbes within the aquatic microbial community and within the BUD wound use quorum sensing communication to upregulate virulence factors or to coordinate other phenotypic behaviors (Reuter, Steinbach, & Helms, 2016). Homoserine lactones are quorum sensing signals produced by many gram-negative bacteria, which can participate in interspecies communication in polymicrobial environments (Tashiro, Yawata, Toyofuku, Uchiyama, & Nomura, 2013). Studies have shown that soil bacteria *Variovorax paradoxus* and *Pseudomonas* strain PAI-A can utilize homoserine lactones as its sole energy source (Huang, Han, Zhang, & Leadbetter, 2003; Leadbetter & Greenberg, 2000). Interkingdom signals such as host-associated hormones (adrenaline, nor-adrenaline, gastrin, insulin and natriuretic peptides) have also been found to promote growth and virulence in bacteria such as Enterohemorrhagic *Escherichia coli*, *Helicobacter pylori, Burkholderia pseudomallei* and *P. aeruginosa* (Andrés Garchitorena et al., 2014; Kendall & Sperandio, 2016). Additional studies showed that *P. aeruginosa* quorum sensing-regulated genes, homoserine lactones and its degradation product can suppress growth and quorum sensing mechanisms in *S. aureus* (Abisado, Benomar, Klaus, Dandekar, & Chandler, 2018; Qazi et al., 2006; Tashiro et al., 2013). Mycolactone is structurally similar to N-acyl homoserine lactone and mycolactone has been shown to increase continuously even after bacterial cultivation reached a plateau in an animal model, suggesting its function in quorum sensing mechanisms (Fred Stephen Sarfo et al., 2013). Hence, studying effects of quorum sensing signaling molecules such as N-acyl homoserine lactone on *M. ulcerans* growth will aid in providing clues of a positive or negative association of *M. ulcerans* with other microorganisms in lesions and in natural environments external to hosts.

Only few studies have been published where *M. ulcerans* RNA was isolated and mycolactone gene expression explored. One study showed that transcription of several key mycolactone biosynthetic genes are driven by a SigA-like promoter (Tobias et al., 2009); however the study did not determine environmental or growth-phase signals that induce mycolactone gene expression. An *in vitro* study showed that *M. ulcerans* downregulated mycolactone in response to various sugar sources (Deshayes et al., 2013). And, one study 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 in stressful conditions (Sanhueza et al., 2019). In this study, we investigated modulation of global and mycolactone gene expressions on exposure to various abiotic and biotic factors to better understand the *M. ulcerans* reservoir, ecology and pathogenesis. First, we tested the effect of UV exposure on mycolactone gene expression to investigate whether mycolactone provides protection to *M. ulcerans* against UV. We also investigated whether mycolactone gene expression and other *M. ulcerans* genes were modulated under changing temperature and oxygen, as these may also be important in *M. ulcerans* stress response, and fitness in aquatic ecosystems. Finally, we measured whether *M. ulcerans* could use insect hormone (Trans-2-hexenyl butyrate) and quorum sensing molecule (N-acyl homoserine lactone) as a sole carbon source. Trans-2-hexenyl butyrate and N-hexanoyl homoserine lactone are signals used for communication by insects and bacteria, respectively (Qazi et al., 2006; Z. M. Zhang, Wu, & Li, 2009). The study on ability of *M. ulcerans* to use these chemical cues from invertebrates and other microbes encountered in natural, aquatic habitats will provide insight on interspecies communication of *M. ulcerans,* which will also aid to understand its reservoir and polymicrobial interaction.

4.3 Materials and methods

4.3.1 Bacterial strain and culture

Mycobacterium ulcerans Parkin or *Agy*99 was inoculated on Middlebrook 7H9 (M7H9) broth containing Oleic Albumin Dextrose Catalase (OADC) and incubated aerobically at 30^0C for 4-6 weeks for use in this study.

4.3.2 Measurement of optical density (OD600)

M. ulcerans form aggregates in culture. Hence, the aggregates were broken by passage through a 20G syringe, followed by a 25 G syringe 10 times each to measure the optical density, using M7H9 as a blank.

4.3.3 Measurement of bacterial growth

M. ulcerans was serially diluted in $\log_{10}$ concentrations on M7H10 agar plates in triplicate using the spread plate technique and incubated at 30°C. Colonies were counted to determine colony forming units/mL after 4-6 weeks to determine the effect of the abiotic and biotic factors on *M. ulcerans* growth . For experiments testing ability of *M. ulcerans* to utilize N-acyl homoserine lactone and insect hormone as a sole source of carbon, colonies were counted, and an average was taken for the triplicates.

4.3.4 Experiment on utilization of insect hormone and N-acyl homoserine lactone as a sole source of carbon

The ability of *M. ulcerans* to utilize trans-2-hexenyl-butyrate (insect hormone) and N-hexanoyl homoserine lactone (quorum sensing molecule) as a sole carbon source was tested by adding these compounds to M7H9 media without oleic albumin dextrose catalase (OADC) and with or without glycerol. The initial inoculum of *M. ulcerans* for the experiment testing the

ability of *M. ulcerans* to utilize N-hexanoyl homoserine lactone and trans-2-hexenyl-butyrate (insect hormone) as a sole carbon were 8 x 10^2 CFU/mL and 2.1 x 10^5 CFU/mL respectively. The *M. ulcerans* culture in these media were then incubated at 30^0C and growth was measured and compared to control (M7H9 containing OADC) for up to 8 weeks. Culture was plated in triplicate, and the average for triplicate growth was recorded. The concentration of N-hexanoyl homoserine lactone was 0.5mg/mL and trans-2-hexenyl-butyrate was 0.1 and 1%. The concentration of N-hexanoyl homoserine lactone was based on a study by Leadbetter *et al.* (2000), where the researcher used basal medium containing N-3-oxohexanoyl-l-HSL (3OC6-HSL) in the concentration of 500 μg · ml−1 for enrichment of bacteria that can utilize quorum sensing molecule as a sole energy source from soil(Leadbetter & Greenberg, 2000). The concentration of Trans-2-hexenyl butyrate was based on its ability to give odor at 1% concentration(Sigma-Aldrich, n.d.).

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

Expression of mycolactone was investigated on exposure to UV for different time intervals (30 s, 2 min and 10 min). The time for UV exposure was determined based on a paper 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 secs and 22 secs UV exposure, respectively(David, Jones, & Newman, 1971). Briefly, *M. ulcerans* during exponentially growth (5.7 x 10^5 CFU/mL, 30 mL) was transferred to a Petri plate 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 a Petri plate, but without UV exposure in the same hood. Each experiment was conducted in at least triplicate sampling and repeated twice.

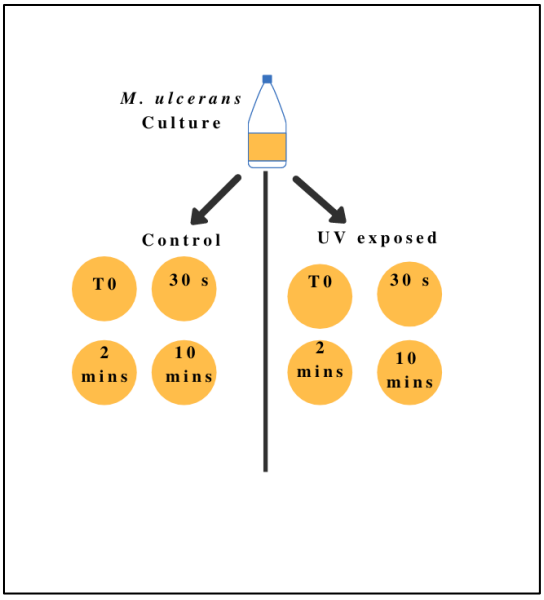

Figure 4.1 Schematic representation of study on effect of UV radiation on *M. ulcerans* growth and mycolactone gene expression.

At each timepoint and condition, 1 mL of sample was transferred for serial dilution and growth measurement, 5.0 mL of sample was transferred for RNA isolation and RT-qPCR to measure mycolactone gene expression.

4.3.6 Effect of temperature and oxygen on *M. ulcerans* growth

To determine the effects of different oxygen conditions on *M. ulcerans* growth, *M. ulcerans* initially grown aerobically was exposed to microaerophilic and anaerobic conditions for 24 hours, by placing those cultures within anaerobic chambers with specific gas paks (BD) and oxygen indicators. After 24 hours the optical density for each sample was measured. The *M. ulcerans* cultures were then placed back in aerobic conditions for an additional 24 hours (48 hours from initial time point). The optical density for each sample was measured. To study the effects of temperature, *M. ulcerans in* exponential growth at 30^0C were exposed to 37^0C and 25^0C for 24 hours and then brought back to 30^0C for an additional 24 hours. The optical density was measured for each time point. Each experiment was conducted with triplicate replicates and at least three times.

4.3.7 Determining whether there was a combined effect of high temperature and low oxygen in mycolactone and global gene expression

Exponentially grown *M. ulcerans* at aerobic, and at 30^0C were exposed to 37^0C under microaerophilic or anaerobic conditions for 24 hours. After 24 hours, the cultures were again brought back to control conditions (30^0C and aerobic) for an additional 24 hours (48 hours from initial timepoint). Each experiment was conducted with triplicate replicates.

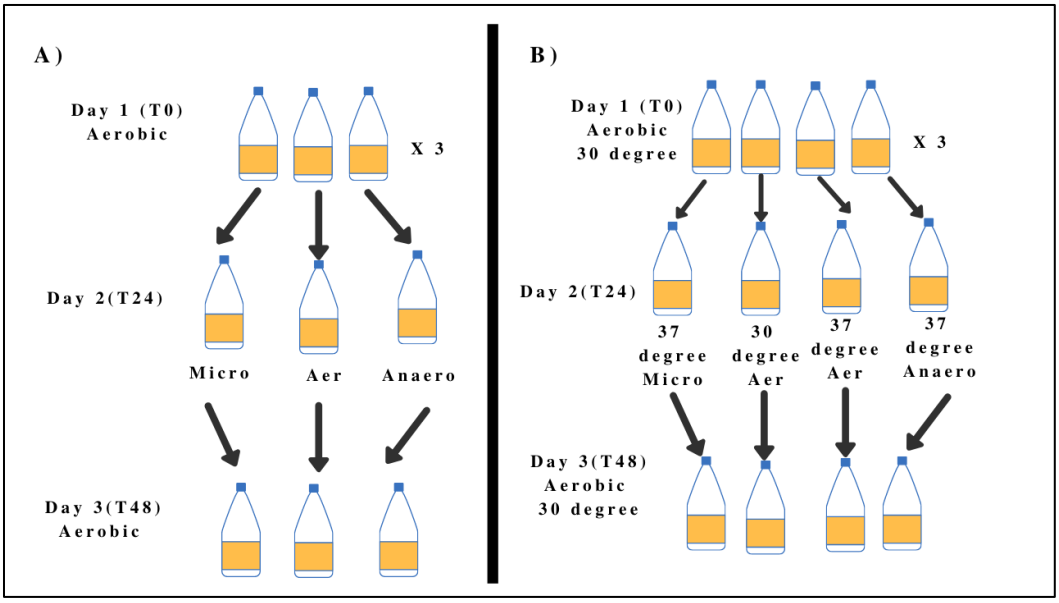

Figure 4.2 Schematic representation of study on the effect of varying oxygen and temperature conditions on *M. ulcerans* growth, and mycolactone and global gene expression

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 global gene expression of *M. ulcerans*. At each time point 5 mL of *M. ulcerans* culture was transferred for RNA isolation. The RNA was converted to cDNA for RT-qPCR targeting ER. The RNAseq analysis was performed to observe the effect of combined high temperature and low oxygen (microaerophilic) conditions on global gene expression

4.3.8 RNA isolation

RNA was isolated using the Trizol method, according to the manufacturer's instructions. Briefly, bacterial cell suspension was centrifuged and 1.0 mL Trizol reagent was added to the

pellet and mixed thoroughly and bead beaded. After incubation for an 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, and integrity verified by gel electrophoresis.

4.3.9 Preparation of cDNA

cDNA was prepared using the Verso enzyme 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.

4.3.10 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 (Jenkin, Stinear, Johnson, & Davies, 2003). The polyphosphate kinase (*ppk*) gene was used as a house keeping gene. 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 using a BioRad CFX96 with parameters that include 95.00C for 10:00 min, and 39 cycles of 95.0 for 0:15, 55.00C for 0:30, 57.00C for 0.30 repeated for 39 times.

4.3.11 Statistical analysis

The significant difference in growth of *M. ulcerans* under the control condition and at conditions exposed to abiotic or biotic stresses were determined using student's T-test in excel. RT-qPCR data were analyzed by relative quantification of gene expression compared to the control using python code implementing the ΔΔCT method (Livak & Schmittgen, 2001). The housekeeping gene used was *ppk* and the target gene was ER. 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. This type of reciprocal transformation to determine up- and downregulation of genes has been described for microarray data analysis by Babu (2004) (Babu, 2004). The significant cut-off value (α) for upregulation and downregulation was 0.05.

4.3.12 RNA seq analysis

RNA libraries were created from combined triplicate replicates of *M. ulcerans* RNA samples under aerobic and 30^0C (T0-T48H), aerobic and 37^0C (T24 H), microaerophilic and 37^0C (T24 H), and samples that were transferred from 37^0C, aerobic or microaerophilic conditions to aerobic and 30^0Cat 48 hr (aerobic and 37^0C (T48 H), microaerophilic and 37^0C (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. Resulting high-quality reads were aligned to the *M. ulcerans Agy*99 reference genome GCF_000013925.1 (downloaded from the NCBI database). 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. Differential expression was measured between treatments against controls in the CLC Workbench. The program uses multi-factorial statistics (Wald test and Likelihood Ratio test) based on a negative binomial generalized linear model ("Introduction to CLC Genomics Workbench (USER MANUAL)," 2017). Treatment reads with a log 2 fold change of 1.5 or higher FDR adjusted p-value less than or equal to 0.05 were considered significant (Ibraim et al., 2019). The top 25 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.).

4.4 Results

4.4.1 *M. ulcerans* cannot utilize insect hormone and quorum sensing molecule as a sole source of carbon

M. ulcerans was exposed to nutrient stress and tested for its ability to utilize biotic factors such as N-hexanoyl homoserine lactone and trans-2-hexenyl butyrate as a sole carbon source. These are carbon rich compounds used as chemical cues by organisms to communicate (trans-2-

hexenyl butyrate by insects and N-hexanoyl homoserine lactone by bacteria). Previous studies have shown that sewage bacteria can utilize chemical signals such as quorum sensing molecules as a sole carbon source (A. L. Kim, Park, Lee, Lee, & Lee, 2014). In this study, the growth of *M. ulcerans* inoculated on M7H9 media containing N-hexanoyl homoserine lactone or trans-2-hexenyl butyrate but without OADC was compared to growth of *M. ulcerans* on M7H9 media containing OADC (control). Beginning on day 2, no *M. ulcerans* growth was observed in the media containing N-hexanoyl homoserine lactone (with and without glycerol) instead of OADC. Similarly, no growth was observed for media containing 0.1% and 1% insect hormone (with and without glycerol) instead of OADC. However, *M. ulcerans* growth was observed in control media containing OADC for both experiments (Figure 4.3).

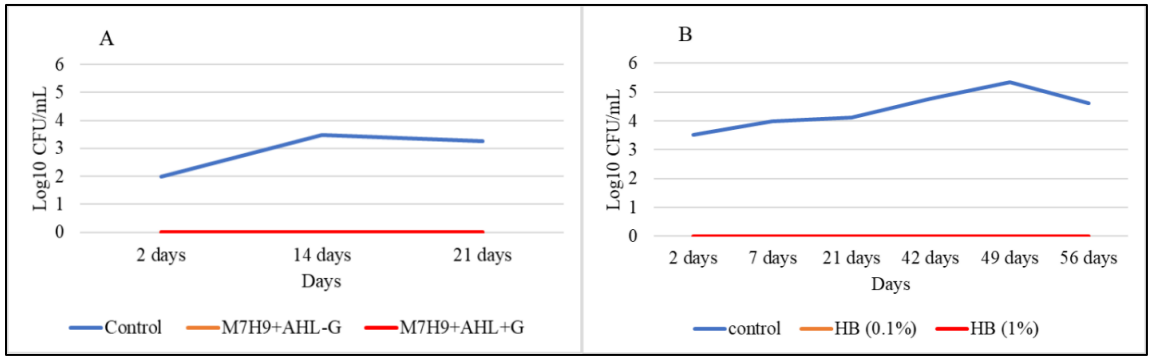

Figure 4.3 Growth of *M. ulcerans* in M7H9 broth containing quorum sensing signal and insect hormone as a sole carbon source

(A) *M. ulcerans* was not able to grow in presence and absence of glycerol (G) when N-acyl-homoserine lactone (AHL) was used as a sole carbon source. In contrast growth was unaffected in M7H9 media containing OADC as a carbon source (control), where colonies were too numerous to count at 28 days. (B) *M. ulcerans* did not grow on M7H9 broth containing trans-2-hexenyl butyrate (HB-insect pheromone) as a sole carbon source but grew on media containing OADC (control).

4.4.2 There was little effect of UV exposure on *M. ulcerans* growth and ER gene expression

No significant difference in *M. ulcerans* growth was observed following exposure to UV for 30 seconds (P=0.18), 2 minutes (P=0.45) and 10 minutes (P=0.10) compared to controls (Figure 4.4). However, ER gene expression was marginally modulated on exposure to UV for 10 minutes (P=0.051). There was negligible effect on mycolactone gene expression upon exposure of *M. ulcerans* to UV for 30 seconds whereas slight downregulation of mycolactone gene expression was observed when *M. ulcerans* were exposed to UV for 2 minutes, although the effects were not statistically significant (Figure 4.5).

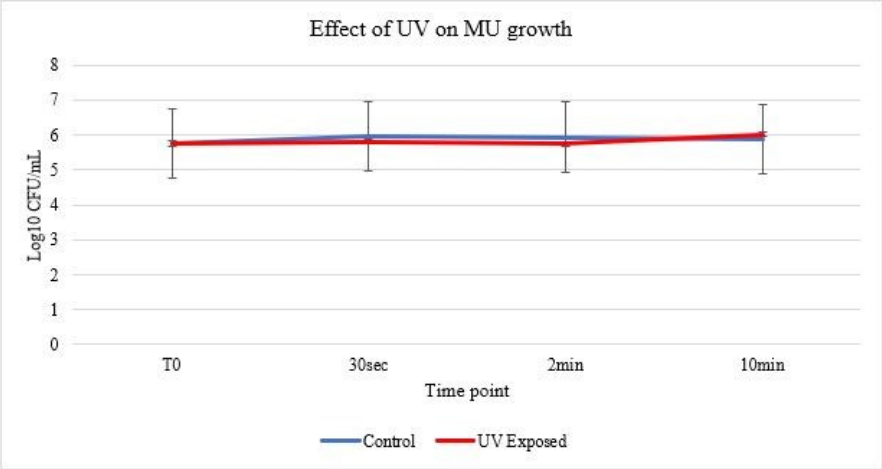

Figure 4.4 Line graph representing effects of UV on *M. ulcerans* growth

The growth of *M. ulcerans* was similar for control (blue line) and *M. ulcerans* exposed to UV for 30 seconds, 2 and 10 minutes (red line).

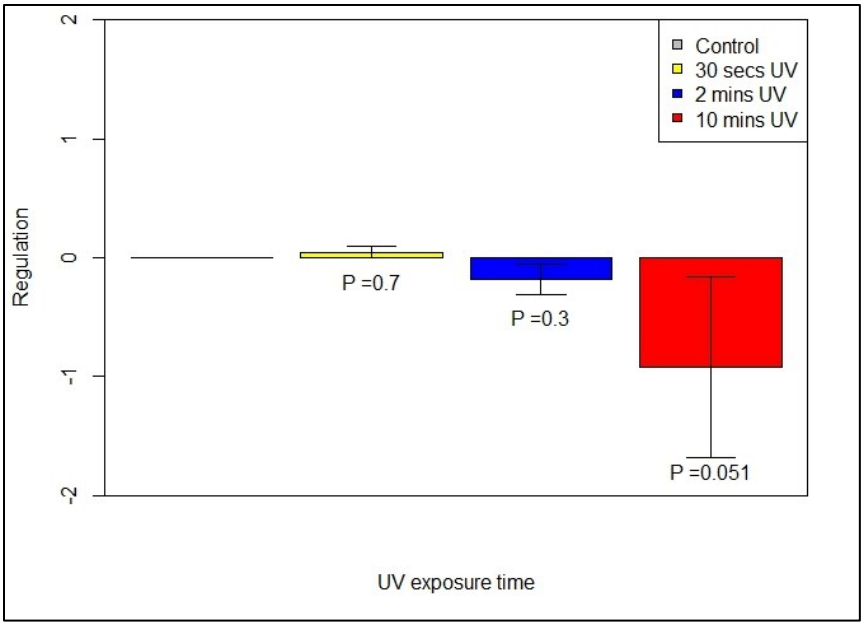

Figure 4.5 Effect of UV exposure on mycolactone expression.

There was negligible effect of 30 seconds UV exposure on mycolactone (ER) expression (yellow bar) and slight ER downregulation for *M. ulcerans* exposed to UV for 2 minutes (blue bar). The exposure of UV for 10 minutes caused downregulation in mycolactone expression with marginal significance (red bar). Results from two separate experiments were combined to obtain final result.

4.4.3 *M. ulcerans* growth and mycolactone expression was modulated when exposed to single and combined environmental stressors (oxygen or temperature)

The effect of environmental stresses including low oxygen, high temperature, combined low oxygen and high temperature on mycolactone gene expression was studied. An exponentially grown *M. ulcerans* was exposed to various temperature and oxygen conditions to understand their impact on *M. ulcerans* growth and mycolactone gene expression. The growth of *M. ulcerans* was not affected when grown on microaerophilic and anaerobic conditions in comparison to aerobic conditions (Figure 4.6A). Similarly, there was no significant difference in *M. ulcerans* growth when subjected to different temperature (30^0 vs 37^0 vs 25^0) for 24 hours (Figure 4.6B). When the cultures were brought back to 30^0, cultures subjected to 37^0 had similar

growth as cultures subjected to 30^0; however, there was a significant increase in growth for cultures subjected to 25^0 compared to culture subjected to 30^0 C (Figure 4.6B).

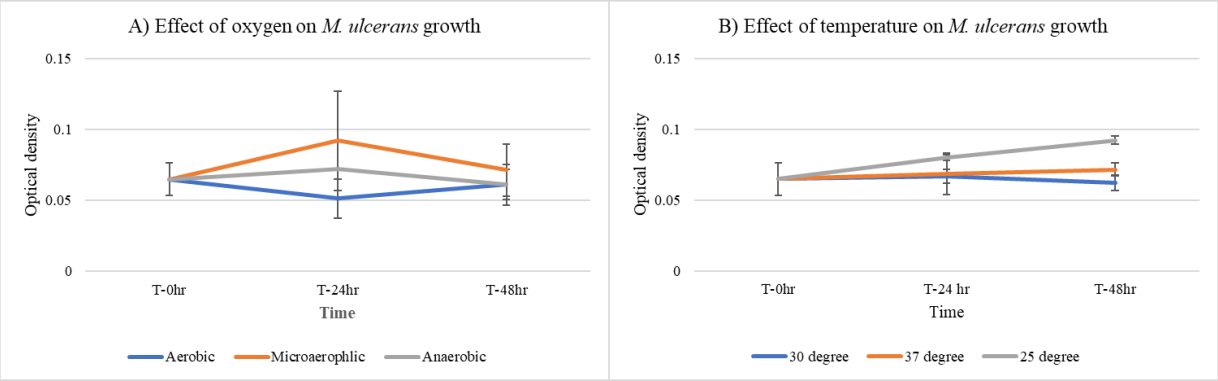

Figure 4.6 Line graph showing optical density measurement of *M. ulcerans* when exposed at different oxygen and temperature conditions

A) Optical density of *M. ulcerans* when exposed to aerobic (blue line), microaerophilic (orange) and anaerobic (grey line) conditions. Exponential *M. ulcerans* initially at aerobic condition 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), 37^0C (orange) and 25^0C (grey line). Exponential *M. ulcerans* initially at 30^0C were exposed to their respective temperature condition for 24 hours and then exposed back to 30^0C for an additional 24 hours (48 hours from initial time point).

The exposure of *M. ulcerans* to microaerophilic conditions for 24 hours showed significant upregulation of ER expression (P=0.0009). Gene expression was slightly downregulated when *M. ulcerans* exposed to microaerophilic conditions were transferred back to aerobic conditions, however, the difference was statistically insignificant. *M. ulcerans* exposed to anaerobic conditions for 24 hours had non-significant upregulation of mycolactone gene expression. Upon transferring the bacteria that were exposed to anaerobic condition for 24 hours back to aerobic conditions, there was a significant (P=0.005) upregulation of mycolactone gene expression compared to control *M. ulcerans* exposed under aerobic conditions during the duration of the 3 day study.

ER gene expression was significantly downregulated when *M. ulcerans* grown aerobically at 30^0C (control) were exposed aerobically to 37^0C (P= 0.02923) and significantly upregulated when brought back to aerobic 30^0C (P=0.0002 compared to control and P=0.001 compared to 37^0C at aerobic condition-day 2). Although ER gene expression for *M. ulcerans* was downregulated when exposed to 37^0C under microaerophilic conditions and upregulated when brought back to 30^0C and aerobic conditions, the regulation was not statistically significant (Figure 4.8). The exposure of *M. ulcerans* at 30^0C and aerobic conditions to 37^0C and anaerobic conditions caused significant downregulation of mycolactone gene expression (P=0.002). When the cultures were brought back to control conditions, ER gene expression was still downregulated (statistically insignificant) compared to control, but it was significantly upregulated (P=0.01) compared to *M. ulcerans* at 37^0C and anaerobic conditions on day 2 (Figure 4.9).

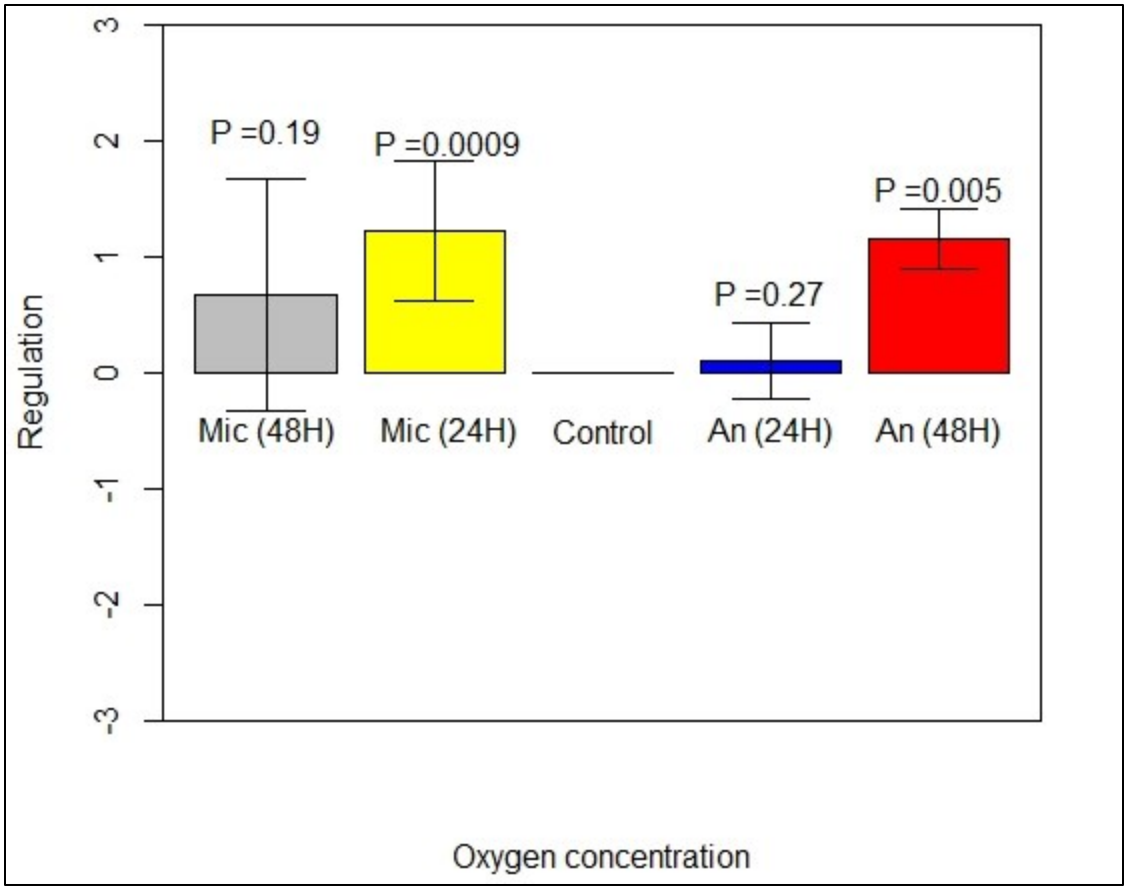

Figure 4.7 Modulation of mycolactone expression (ER) on exposure to different oxygen conditions.

Exposure of *M. ulcerans* to a microaerophilic environment for 24 hours caused significant upregulation (P=0.0009) of mycolactone expression (yellow bar) and transferring the bacteria back to aerobic condition led to slight downregulation in mycolactone expression (grey bar). Exposure of *M. ulcerans* to anaerobic condition for 24 hours led to slight mycolactone gene upregulation (blue bar), but transfer back to aerobic condition caused significant ER upregulation (P=0.005) (red bar) compared to control *M. ulcerans* exposed to aerobic condition during the entire 3 day experiment. Control: *M. ulcerans* exposed to aerobic condition at 0, 24 and 48 hours; Mic (24 H): *M. ulcerans* exposed to microaerophilic condition for 24 hour; Mic (48H): following transfer from microaerophilic condition to aerobic condition for an additional 24 hours (48 hours from the initial time point)); An (24H): *M. ulcerans* exposed to anaerobic condition for 24 hours; An (48H)-following transfer back to aerobic condition for an additional 24 hours (48 hours from the initial time point)). Presented are results from combined experiments, with standard deviations.

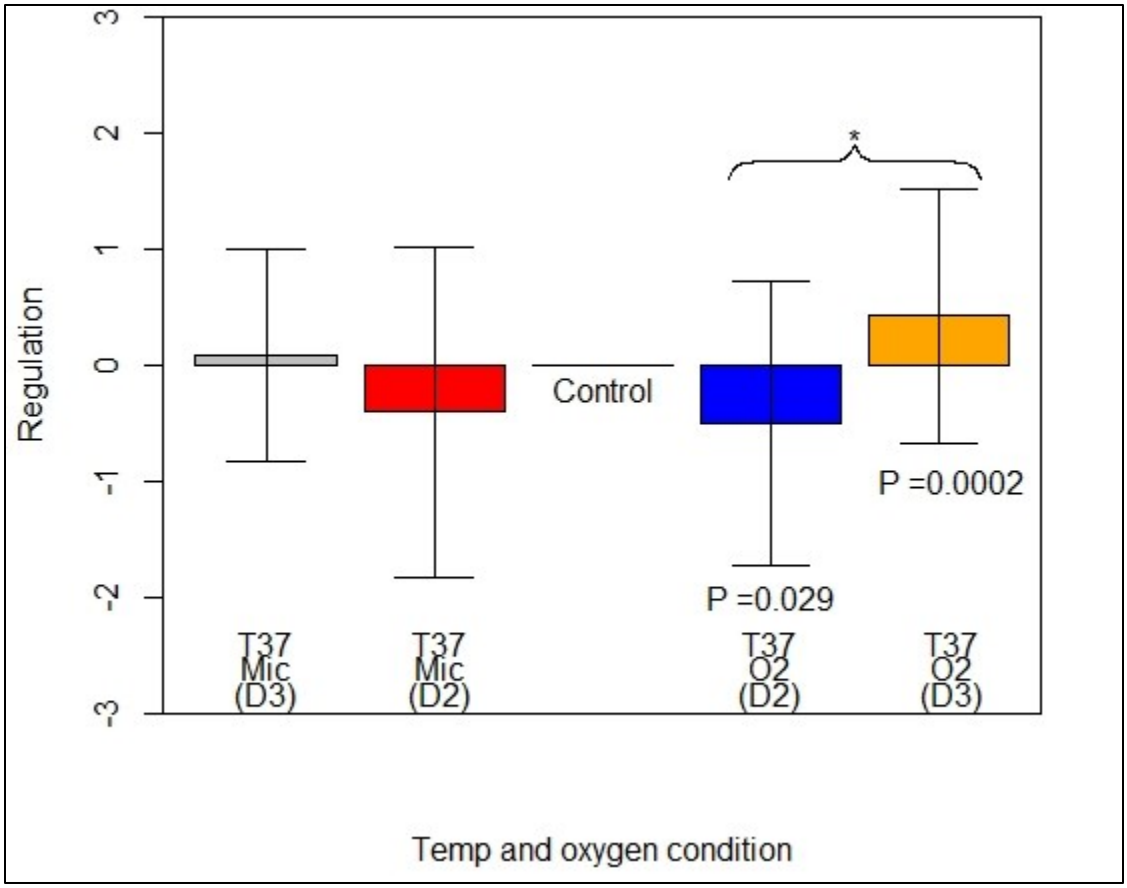

Figure 4.8 Modulation of ER expression after exposure to high temperature and low oxygen conditions.

M. ulcerans exposure to high temperature (37^0C) caused significant downregulation of ER gene expression (blue bar). Similarly, ER expression was downregulated on exposure to combined high temperature and low oxygen (37^0C and microaerophilic) conditions (red bar) but it was not statistically significant. When *M. ulcerans* culture exposed to 37^0C and microaerophilic condition was transferred back to 30^0C and aerobic conditions (grey bar), ER gene expression was upregulated, however, was statistically insignificant. In contrast, when aerobic *M. ulcerans* exposed to 37^0C was transferred back to aerobic, 30^0C conditions, (orange bar), ER was significantly upregulated compared to control (P=0.0002), and *M. ulcerans* exposed at 37^0C on day 2 (represented by *, P= 0.001). T30-O2-D1-3: *M. ulcerans* at 30^0C and aerobic conditions from days 1-3); T37-O2-D2: *M. ulcerans* exposed to 37^0C and aerobic conditions on Day 2); T37-Mic-D2: *M. ulcerans* exposed to 37^0C at microaerophilic condition on day 2); T37-Mic-D3: *M. ulcerans* at 37^0C and microaerophilic conditions on day 2 that were transferred to 30^0C and aerobic condition on day 3; T37-O2-D3: *M. ulcerans* at 37^0C and aerobic conditions at day 2 but transferred back to 30^0C and aerobic conditions on day 3).

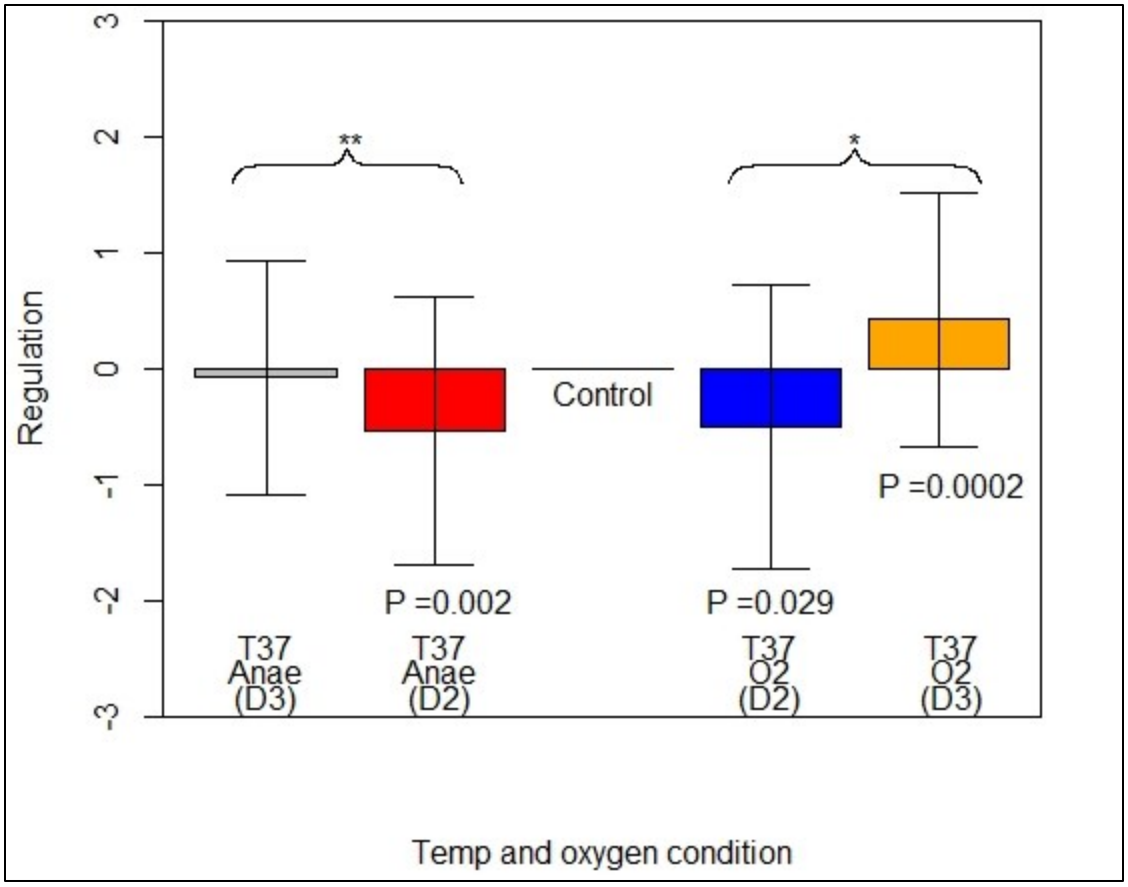

Figure 4.9 Modulation of ER expression after exposure to high temperature and low oxygen conditions (Anaerobic).

The blue bar represents *M. ulcerans* at 37^0C under aerobic conditions on day 2 and the orange bar represents *M. ulcerans* when brought back to 30^0C on day 3. ER gene expression was significantly downregulated on exposure to combined high temperature and low oxygen (37^0C and anaerobic) conditions (red bar). When *M. ulcerans* culture exposed to 37^0C and anaerobic condition was brought back to 30^0C and aerobic conditions (grey bar), ER gene expression was significantly upregulated compared to *M. ulcerans* at 37^0C and anaerobic conditions on day 2 (represented by **, P=0.01). However, the upregulation was not statistically significant compared to control *M. ulcerans* grown at 30^0C and aerobic conditions from days 1-3). T30-O2-D1-3: *M. ulcerans* at 30^0C and aerobic conditions from day 1-3); T37-O2_D2: *M. ulcerans* exposed to 37^0C and aerobic conditions on Day 2; T37-An-D2: *M. ulcerans* exposed to 37^0C and anaerobic conditions on day 2; T37-An-D3: *M. ulcerans* at 37^0C and anaerobic on day 2 but brought back to 30^0C and aerobic conditions on day 3; T37-O2-D3: *M. ulcerans* at 37^0C and aerobic conditions at day 2 but transferred to 30^0C and aerobic conditions on day 3.

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

Exponentially grown *M. ulcerans* at 30^0C under aerobic conditions was exposed to higher temperature (37^0 C) and the combination of high temperature and low oxygen condition (37^0C and microaerophilic condition). RNAseq analysis was performed to determine the regulated genes as a stress response. Principal component analysis showed a clear separation in global gene expression between triplicate controls (*M. ulcerans* at 30^0C and aerobic condition from days 1-3), *M. ulcerans* exposed to 37^0C under aerobic conditions on day 2 (T37-O2-D2 (GE)) and *M. ulcerans* exposed at 37^0C and microaerophilic condition on day 2 (T37.Mic-D2 (GE)) (Figure 4.10). There was a higher difference in global gene expression between control and (T37.Mic-D2 (GE)) samples than between control and (T37-O2-D2 (GE)) suggesting a greater effect of combined stress of high temperature (37^0) and low oxygen (microaerophilic) conditions in global gene expression. When *M. ulcerans* was brought back to control conditions (30^0C and aerobic condition) on day 3, global gene expression became more similar to control (T30-O2-D1-3).

A heatmap for the top 25 significantly regulated genes (p values less than or equal to 0.05) showed that 3 genes were downregulated, whereas 22 genes were upregulated in *M. ulcerans* transferred from control conditions to 37^0C and under microaerophilic conditions for 24 hours, compared to controls maintained during the entire 3 day experiment. Similarly, 2 genes were downregulated whereas 22 genes were upregulated, and 1 gene was unaffected for *M. ulcerans* at 37^0C and aerobic condition compared to control. Most of these genes were found in the chromosome with the exception of two genes, MUL_RS00210 and MUL_RS00170, corresponding to *mls*A2 and *mls*B, respectively that are found on the pMUM001 plasmid of *M. ulcerans* (T. P. Stinear et al., 2004). For most of these genes, higher expression was observed

(represented by brighter red color) for *M. ulcerans* at combined stress of high temperature (37^0C) and low oxygen (microaerophilic) conditions compared to *M. ulcerans* exposed only to the single, high temperature stress (Figure 4.11). When *M. ulcerans* at higher temperature (37^0) and lower oxygen (microaerophilic) conditions on day 2 were brought back to aerobic and 30^0C on day 3, there was an upregulation of mycolactone expression (*mls*A2 and *mls*B) (brighter color).

Figure 4.10 Principal Component Analysis showing global gene expression at different temperature and oxygen conditions.

M. ulcerans samples exposed to higher temperature (37^0C) and low oxygen condition (microaerophilic) were separated from control (30^0C and aerobic condition) samples. The combined impact of double stress (high temperature and microaerophilic) on *M. ulcerans* global gene expression was higher compared to impact of single stress (high temperature), as indicated by distance from control. However, the differences in the gene expression was reduced when *M. ulcerans* was brought back to 30^0C under aerobic conditions on day 3. T37-O2-D2 (*M. ulcerans* exposed to 37^0C and aerobic conditions on day 2), T37-Mic-D2 (*M. ulcerans* exposed to 37^0C under microaerophilic conditions on day 2), Each triplicate was brought back to 30^0C and aerobic conditions on day 3, as indicated by T37-O2-D3 and T37-Mic-D3. T30-O2-D1-3 were triplicate samples grown at 30^0C under aerobic conditions during the entire 3 day experiment.

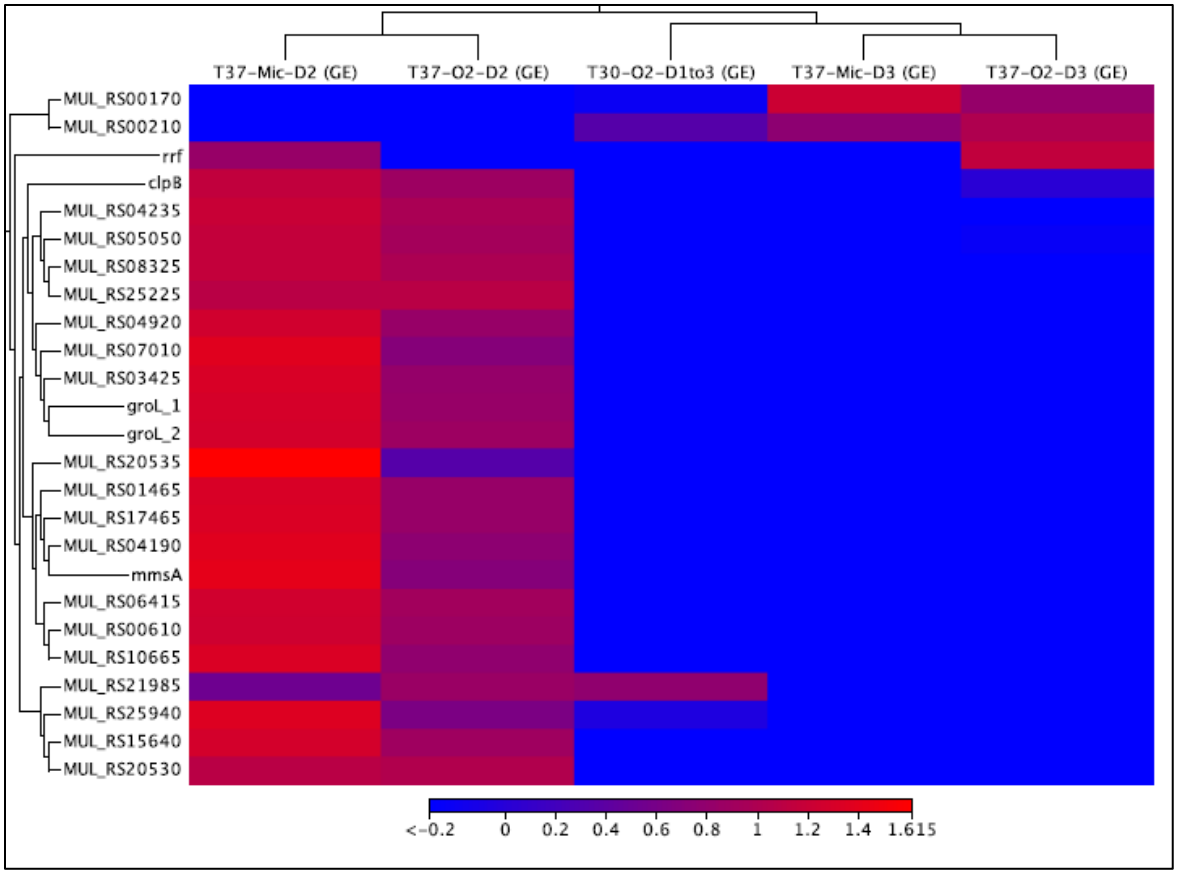

Figure 4.11 Heat map showing the top 25 differentially expressed genes when *M. ulcerans* were exposed to stress condition (high temperature and low oxygen condition).

Exposure to high temperature caused upregulation of 22 genes and down regulation of 2 genes whereas exposure to combined high temperature and low oxygen condition (microaerophilic) caused upregulation of 22 genes and downregulation of 3 genes. The upregulated genes included stress response genes such as *hsp*, *clp*B, *gro*S, *dna*K and *ahp*D. The upregulation of these genes was higher when exposed to double stress of high temperature and low oxygen compared to single stress of high temperature (indicated by brighter color). The gene expression profile of *M. ulcerans* when brought back to normal (30^0C and aerobic condition) was similar to control. The labels for the heat map are similar to labels for principal component analysis.

Among the downregulated genes for single and double stress conditions (MUL_RS00170, MUL_RS00210 and MUL_RS21985), two were related to metabolism whereas one was an uncharacterized protein. The two downregulated genes represented type I modular polyketide synthase genes, *mls*B and *mls*A2, respectively, that are partially responsible for mycolactone production (T. P. Stinear et al., 2004). Similarly, the upregulated genes included 8 genes that were related to metabolism, 8 genes related to genetic information processing, 5 genes related to environmental information processing /signaling and cellular processes, and 3 genes related to human diseases. The upregulated metabolic genes were MUL_0753, MUL_4884, *acc*D6, *ahp*D, MUL_0744, *mms*A, *mas* and *eth*A. These genes were responsible for metabolism of lipids, carbohydrate, amino acid, enzymes as well as xenobiotic degradation (Table 1). Similarly, genes involved in genetic information processing were *rrf*, *clp*B, *gro*S, *sig*B, *whi*B5, *dna*K, *gro*L1 and *gro*L2. Among these genes, *clp*B and *gro*S encode chaperones and folding catalysts, *sig*B and *whi*B5 function in transcription and *dna*K, *gro*L1 and *gro*L2 are involved in folding, sorting and degradation. Genes *dna*K, *gro*L1 and *gro*L2 are also involved in signaling and cellular processes (exosomes) and human diseases in *M. tuberculosis* (Lupoli, Fay, Adura, Glickman, & Nathan, 2016; Stapleton, Smith, Hunt, Buxton, & Green, 2012). Other genes related to environmental information processing/signaling and cellular processes were MUL_0911 and *hsp20*. Gene MUL_0911 is related to integral membrane transport protein and *hsp* is related to Heat shock protein. Genes *hsp*20, *clp*B, *gro*S, *dna*K and *ahp*D are also stress response genes (Table 1).

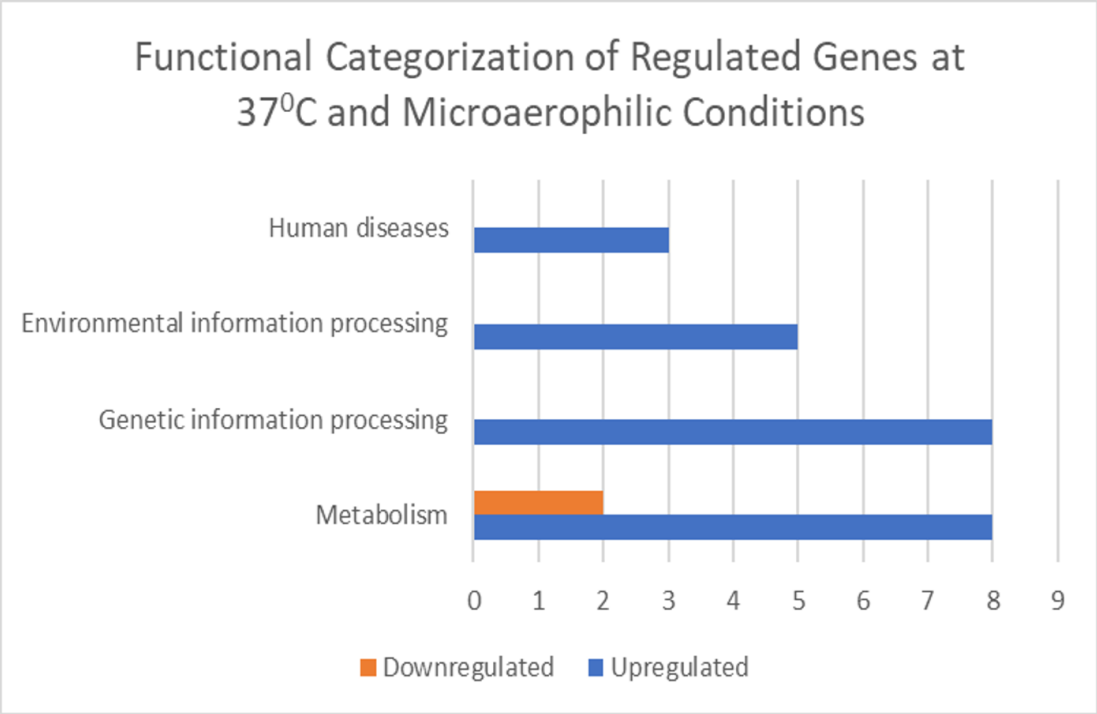

Figure 4.12 Functional categorization of differentially expressed genes of *M. ulcerans* when exposed to high temperature (37^0C) and low oxygen conditions (microaerophilic).

The two downregulated genes (orange color) related to metabolism were *mls*B and *mls*A2, that are partially responsible for mycolactone synthesis. The upregulated genes (blue color) were related to several functional categories (metabolism, genetic information processing, environmental information processing/ signaling and cellular processes and human diseases). Some of the genes such as *dna*K, *gro*L1 and *groL*2 were included in 3 functional categories (genetic information processing, signaling and cellular processes and human diseases (*M. tuberculosis*)).

4.5 Discussion

This is among one of the first studies investigating *M. ulcerans* growth and modulation of mycolactone and global gene expression in response to abiotic conditions, in an effort to elucidate the *M. ulcerans* environmental niche and factors promoting *M. ulcerans* pathogenesis. Exposure of *M. ulcerans* to UV caused time-dependent downregulation of ER gene expression, but not on growth. There was a marginally significant downregulation (P=0.051) of ER expression when *M. ulcerans* was exposed to UV radiation for 10 mins. This contrasted with our initial prediction that mycolactone would be upregulated on exposure to UV and play a role to provide protection to *M. ulcerans* against UV exposure. Lagha (2012) showed that exposure of UV caused downregulation of toxin regulating genes (*tox*S and *tox*R) in another environmental pathogen *V. parahaemolyticus* (Lagha, 2012). UV exposure can damage DNA and may require upregulation of genes needed for its repair. The upregulation of these stress response gene could occur at the expense of mycolactone expression. However, the color of UV treated *M. ulcerans* colonies were yellow on M7H10 agar plates indicating mycolactone production. In our experiment, *M. ulcerans* culture was exposed to UV radiation for a short duration of time (up to 10 mins). Even though this duration of UV exposure caused downregulation of mycolactone (ER) expression, it may not completely shut down its synthesis and thus there could possibly still be mycolactone production that can cause formation of pigmented colonies on M7H10 agar plates. The quantitation of mycolactone production (work in progress) will further elucidate the effect of UV on mycolactone production. Premature termination of the *crt*I gene in *M. ulcerans* that protects its progenitor *M. marinum* against sunlight damage suggested *M. ulcerans* either resides in UV protected areas or there is presence of other machineries to counteract the damage (Demangel et al., 2009; T. P. Stinear et al., 2000). However, in our study, *M. ulcerans* growth

was not affected by UV radiation. However, our study included exposure of *M. ulcerans* to UV radiation for a short duration (10 mins) and further investigations on exposing *M. ulcerans* to longer duration is needed to confirm its resistance to UV radiation long-term. Although the present study showed downregulation of mycolactone (ER) expression on UV exposure, this was not significant. Further studies on transcriptional analysis is also required to understand regulation of other machineries that can confer photoprotection and/or DNA repair to protect *M. ulcerans* against UV damage.

M. ulcerans was not able to use N-hexanoyl homoserine lactone and trans-2-hexenyl butyrate (0.1% and 1%) as sole carbon sources. A few studies have investigated the effect of nutrient sources on *M. ulcerans* growth and gene expression (Deshayes et al., 2013; Sanhueza et al., 2016, 2019). Nutrients such as glucose and chitin have been shown to enhance *M. ulcerans* growth (Deshayes et al., 2013; Sanhueza et al., 2016). Glucose caused upregulation of mycolactone gene expression but decrease in mycolactone production (Deshayes et al., 2013). Similarly, other nutrients such as chitin and calcium can modulate expression of genes related to DNA replication and metabolic pathways respectively (Sanhueza et al., 2019). Mycolactone is structurally similar to N-acyl homoserine lactone and a study in later chapters has shown that mycolactone downregulates Agr quorum sensing system components in *S. aureus. M. ulcerans* has been detected in water bugs from families Belostomatidae and Naucoridae (Andrés Garchitorena et al., 2014; Marion, Chauty, et al., 2014). Hence, in this study we tested if *M. ulcerans* can utilize the interspecies (bacteria) and interkingdom (insects) signaling molecules as sole carbon sources. For the tested concentration, *M. ulcerans* was not able to utilize these molecules as a sole carbon source. Further, there was no significant regulation of mycolactone (ER) gene expression in the presence of the quorum sensing molecule (data not shown).

However, concentration gradients should be investigated to understand their impact on *M. ulcerans* growth and gene regulation.

Microorganisms are equipped with machineries to counteract against adverse environmental conditions in their natural habitat and during host. Changes in environmental conditions such as higher temperature and lower than optimal oxygen conditions can upregulate several stress response genes in an environmental pathogen that can aid in the bacterial survival and pathogenicity inside the human host. Production of heat shock protein is one of the stress responses that bacteria mediate to prevent protein unfolding and its functional disruption caused by higher temperature (Pidot et al., 2010). Similarly, low oxygen can slow or cease several cellular processes and hence require upregulation of genes encoding several enzymes to adapt to the environment (Muttucumaru, Roberts, Hinds, Stabler, & Parish, 2004). Thus, microorganisms selectively upregulate or downregulate genes to work in an energy efficient manner and adapt to the changing environment. This study on the regulation of *M. ulcerans* global gene expression at high temperature (37^0C) and low oxygen may provide clues not only toward *M. ulcerans* adaptation to a changing aquatic environment, but also toward mechanisms regulating its pathology upon entry to human body.

Genes encoding transcriptional regulators such as SigB and WhiB5 were upregulated. Sigma factors play an important role during initiation of RNA transcription by aiding binding of RNA polymerase to the RNA promoter (Davis, Kesthely, Franklin, & MacLellan, 2017). Housekeeping sigma factor (e.g. SigA) functions in transcription during growth whereas alternative sigma factors (e.g. SigB) play roles in adaptive responses (Hu & Coates, 1999; Mauri & Klumpp, 2014; Paget, 2015). Sigma B is one of the principal sigma factors and is considered a general stress response in mycobacteria (Manganelli, Voskuil, Schoolnik, & Smith, 2001;

Pettersson et al., 2015). It is upregulated in heat, microaerobic and during nutritional starvation conditions in *M. tuberculosis* and *M. marinum* (Hu & Coates, 1999; Pettersson et al., 2015). The response is especially high during heat stress for *M. tuberculosis* (Hu & Coates, 1999). This sigma factor is regulated by the two component system MprAB and sigma factors E and H under stress conditions (Manganelli et al., 2002, 2001; Pang et al., 2007). The control of *sig*B by these regulons is stress dependent as its control by MprAB and *sig*E is observed for Sodium dodecyl sulfate (SDS) stress, whereas it is controlled by *sig*H on heat stress(Manganelli et al., 2002, 2001; Pang et al., 2007). There is no control of *sig*E on *sig*B expression during heat stress whereas *sig*H control on *sig*B is indirect (Manganelli et al., 2002, 2001). SigH also positively regulates *hsp* and *clp*B genes that are upregulated in our study(Manganelli et al., 2002). SigB 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*(J. H. Lee, Karakousis, & Bishai, 2008). WhiB proteins are redox sensing transcriptional regulators (Casonato et al., 2012). Most of these WhiB proteins interact with *sig*A for transcriptional regulation (Feng, Chen, Wang, Hu, & Chen, 2016). However, the regulation of WhiB5 is not dependent on interaction with *sig*A or *sig*B (Feng et al., 2016). WhiB5 positively regulates 58 genes including type VII secretion systems (ESX-2 and 4) (Casonato et al., 2012). WhiB5 proteins in *M. tuberculosis* are relatively stable and under-expressed in aerobic conditions but are slightly upregulated at 0% oxygen (Larsson et al., 2012). They are suggested to be immunomodulators and enhancers of *M. tuberculosis* survival during nutrient limitation(Casonato et al., 2012). Thus, upregulation of these transcriptional regulators suggests that genes related to stress response and virulence are upregulated on exposure of *M. ulcerans* to body temperature and hypoxic environments inside the human host, which enhances its survival,

persistence and pathogenicity inside the host. Alternatively, these may be evolutionarily conserved mechanisms for stress response in the *M. ulcerans* natural environment.

Several genes related to stress response such as *hsp*20, *clp*B, *gro*S (*gro*ES), *dna*K and *ahp*D were upregulated on exposure of *M. ulcerans* to higher temperature (37^0C) and low oxygen condition (microaerophilic) conditions. Hsp20 is a heat shock protein within an alpha crystallin family("GenomeNet," n.d.). Genes *clp*B, *gro*ES and *dna*K encode chaperones that provide stress response against heat shock (Legname et al., 1996; Pettersson et al., 2015). Stress can cause protein denaturation and thus chaperonins that provide protein folding for protection are upregulated (Qamra, Mande, Coates, & Henderson, 2005). The *clp*B gene is related to virulence in several gram positive (e.g. *S. aureus*) and negative pathogens (e.g. *Salmonella enterica serovar typhimurium*) (Tripathi, Singh, Kumari, Hakiem, & Batra, 2020). In *M. tuberculosis*, *clp*B enhances biofilm formation and promotes survival against hypoxia, heat stress and inside macrophages (Tripathi et al., 2020). Similarly, *dna*K provides protection against heat shock and oxidative stress inside the macrophage to *M. tuberculosis* (Raman et al., 2001). Limited oxygen upregulates the *dna*K protein in *M. bovis* (Srivastava, Saxena, & S. Srivastava, 2018). Gene *ahp*D encode alkyl hydroperoxide reductase that provide stress response in hypoxic environment (Muttucumaru et al., 2004). GroES and GroEL are chaperones that provide protein folding in an ATP dependent manner (Qamra et al., 2005). In mycobacteria, there are two copies of GroEL in contrast to the presence of only 1 copy in *E. coli* (Sharma et al., 2016). GroES and GroEl2 are required whereas GroEL1 may not be needed for survival of *M. tuberculosis* under normal conditions (Hu et al., 2008). GroEL1 is important for stress response against high temperature and low oxygen condition (Hu et al., 2008; Sharma et al., 2016). In a study by Sharma *et al*. (2015), an upregulation of GroEL1 was observed for *M. tuberculosis* in a hypoxic

environment (Sharma et al., 2016). Additionally, a GroEL1 mutant had compromised growth under low oxygen conditions, thereby suggesting its role in providing a fitness advantage to *M. tuberculosis* in a low oxygen intracellular environment (Sharma et al., 2016). GroES and GroEL are also upregulated in *M. bovis* during hypoxia (Srivastava et al., 2018). Similarly, Hu *et al.* (2008) observed defective growth of a *groEL1* mutant *M. tuberculosis* at high temperature (55^0C) (Hu et al., 2008). And, GroEL1 protein plays role in mycolate synthesis during maturation of biofilm in *M. smegmatis* (Ansari & Mande, 2018; Ojha et al., 2005). Proteins ClpB, GroES, GroEL1, DnaK and AhpD are found in the extracellular matrix (ECM) of *M. ulcerans* biofilm (Marsollier et al., 2007), and *M. ulcerans* with ECM have higher colonization and virulence ability (Marsollier et al., 2007). Hence, upregulation of these genes suggests that heat and limited oxygen supply trigger stress response mechanisms in *M. ulcerans* that protect the bacterium against higher body temperature and hypoxic environments during infection, or within polymicrobial communities in its natural environment. Further, these genes could promote biofilm formation and enhance colonization and virulence activity of *M. ulcerans.*

Low oxygen changes metabolism and biomass composition in mycobacteria (Fang, Wallqvist, & Reifman, 2012). Genes related to lipid metabolism are upregulated and metabolism shifts toward low ATP requiring pathways (Fang et al., 2012; Sharma et al., 2016). This can provide an alternative source of carbon (such as fatty acid) during stress (Srivastava et al., 2018). Further, a reverse pathway for conversion of pyruvate to propionyl CoA is also present in *M. tuberculosis* (Serafini et al., 2019). In this alternative short pathway, pyruvate is converted to methylmalonate semi-aldehyde by an unknown mechanism, which is then converted to propionyl-CoA by Methylmalonate semialdehyde dehydrogenase (MmsA) enzyme (Serafini et al., 2019). This propionyl-CoA then converts to fatty acids required for the *M. tuberculosis* cell

envelope (Serafini et al., 2019). The changes in biomass composition mainly include an increase in cell-wall components that cause thickening of the cell wall (Fang et al., 2012). In our experiment, the combined stress of temperature and oxygen upregulated genes related to lipid metabolism (Acyl-CoA dehydrogenase) and mycolic acid synthesis (*acc*D6, *mas*, *eth*A). Similar upregulation of acyl-CoA dehydrogenase (FadE5) was observed for *M. bovis* in an oxygen limited environment (Srivastava et al., 2018). AccD6 (acetyl-CoA carboxyltransferase) is involved in the initial step of mycolic acid synthesis (Pawelczyk et al., 2011). In *M. bovis,* it is shown to be upregulated in low oxygen conditions (Srivastava et al., 2018). Similarly, the *mas* gene encodes mycocerosic acid, a cell wall component and has been shown to be upregulated in microaerophilic conditions in *M. tuberculosis* (Muttucumaru et al., 2004). Another upregulated gene, *eth*, encodes oxidoreductase that oxidizes keto-mycolic acid to synthesize waxy mycolic acids (Ang et al., 2014). 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. Further, the 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. Similar to our study, Muttucumaru *et al*. (2004) also observed upregulation of genes related to oxidoreductases, acyl-CoA dehydrogenase, integral membrane transport protein, monooxygenase, heat shock protein and alkyl hydroperoxide reductase (*ahp*D) for *M. tuberculosis* in hypoxic environments (Muttucumaru et al., 2004).

Interestingly, *mls*A2 and *mls*B genes that are responsible for mycolactone synthesis were downregulated under combined stresses of higher temperature and lower oxygen conditions. Gene *mls*A2 is responsible for the synthesis of the core lactone ring whereas gene *mls*B is responsible for synthesis of the acyl side chain (T. P. Stinear et al., 2004). This contrasts with our

initial hypothesis that predicted upregulation of mycolactone in response to combined effects of high temperature and low oxygen conditions that mimics the sub dermal environment during human infection. 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) (W. Wang, Winlove, & Michel, 2003). The sudden exposure to higher temperature and lower oxygen conditions could induce stress response mechanisms in *M. ulcerans.* Hence, this may cause it to use its energy efficiently for production of enzymes and proteins to cope with damages caused by these stresses thereby compromising mycolactone synthesis. Deshayes *et al.* (2013) observed that *M. ulcerans* compromises its mycolactone production to increase mycobactin production (an iron siderophore) in glucose medium (Deshayes et al., 2013). However, the decrease in mycolactone production was not observed at the transcriptional or translational level, but was suggested to be related to common translocation machinery for both products (Deshayes et al., 2013). Further, mycolactone could provide protection to *M. ulcerans* against oxidative stress as it was upregulated when bacteria were transferred from the low-oxygen environment to an aerobic condition. This has been found in other bacteria, where, for instance, in *S. aureus,* carotenoid production yields bacterial pigment that provides protection against oxidative stress and phagocytic killing (G. Y. Liu et al., 2005). And, loss of genes for carotenoid synthesis led to *M. smegmatis* having increased sensitivity to hydrogen peroxide (Provvedi et al., 2008). Although our results suggest that *M. ulcerans* regulates mycolactone synthesis and other genes in response to the environment, further studies are required to understand the impact on mycolactone production and virulence of *M. ulcerans*. It is suggested that *M. ulcerans* initially turns off mycolactone synthesis to allow intracellular growth but turns it on later to lyse the cell and facilitate the exit of bacteria to

become extracellular in the host (Torrado et al., 2007). Based on our results, the combined effect of high temperature and low oxygen could be signals triggering the turning off of mycolactone synthesis. Additionally, in this study we defined higher temperature and lower than optimal 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 bacteria in its natural habitat and *M. ulcerans's* response to fluctuating temperature and oxygen conditions may be simply a "response" instead of "stress response".

RT-qPCR targeting the ER gene found on the pMUM001 plasmid responsible for mycolactone synthesis showed significant upregulation under microaerophilic conditions. The higher expression of the ER gene in microaerophilic conditions can be related to *M. ulcerans* association with slow moving water with low oxygen levels (Merritt et al., 2010). Expression of the ER gene was downregulated when brought back to aerobic conditions. Similarly, there was significant downregulation of ER when *M. ulcerans* at aerobic and 30^0C was moved to 37^0C under aerobic conditions. Gene expression was significantly upregulated when brought back to aerobic conditions and 30^0C. Although there was downregulation of the ER gene on exposure to combined microaerophilic and 37^0C conditions, it was not statistically significant. But the downregulation of mycolactone gene expression on combined high temperature (37^0C) and microaerophilic conditions shown by RT-qPCR and transcriptome analyses indicates a higher impact of temperature on the downregulation. One of the possible explanations for this is that the microaerophilic conditions could cause upregulation of mycolactone expression, but the combined stress of high temperature and low oxygen could prioritize the expression of other essential genes thereby compromising mycolactone synthesis. In *Vibrio cholerae*, the promoter of cholera toxin regulator (*tox*R) and heat shock (*htp*G) genes overlap but are transcribed in

opposite directions (Parsot & Mekalanos, 1990; Slauch, Taylor, & Maloy, 1997). Hence, increase in temperature allows transcription of *htp*G gene with reduction in *tox*R expression. Further, 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 (T. P. Stinear et al., 2004). 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 RNA-seq results for mycolactone expression.

The exposure to *M. ulcerans* to anaerobic conditions caused slight upregulation of ER gene expression, however, was statistically insignificant. When *M. ulcerans* exposed to anaerobic conditions for 24 hours were brought back to aerobic conditions (additional 24 hours), ER gene expression was significantly upregulated. The combined anaerobic and 37^0C caused significant downregulation of the ER gene. The expression 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 in an aerobic or microaerophilic environmental niche (Demangel et al., 2009). However, its association with mammalian feces and asymptomatic gut colonization raises the questions on its lack of pathogenicity in the anaerobic intestinal environment (Carson et al., 2014; Janet A.M. Fyfe et al., 2010; Lavender et al., 2008; C. R. O'Brien et al., 2014; Röltgen et al., 2017; Tian et al., 2016). Although the anaerobic (single stress) condition does not affect mycolactone gene expression, combining a high temperature and anaerobic conditions could downregulate mycolactone gene expression and may account for the lack of pathogenesis in the mammalian intestine. Indeed, oxygen and carbon dioxide concentration has shown to impact production of toxic shock syndrome toxin 1 (TSST-1) toxin in *S. aureus* (A. C. L. Wong & Bergdoll, 1990; Yarwood & Schlievert, 2000). An efficient

TSST-1 toxin (μg/10^{10} CFU) production was observed for an increase in CO_2 concentration for *S. aureus*. The shift from anaerobic to increasing oxygen concentrations (2%) caused increase in toxin production; however, was decreased for increasing oxygen concentration (6%) in an environment without CO_2 (Yarwood & Schlievert, 2000). 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.

It has been suggested that mycolactone producing mycobacteria (MPM) such as *M. ulcerans* have evolved from a common *M. marinum* progenitor by undergoing various gene deletions, pseudogene formation and rearrangement, and acquiring plasmid pMUM001 to adapt to a specific ecological niche (Yip et al., 2007). These gene deletions and pseudogenes formation suggests that *M. ulcerans* occupies a restricted and narrow niche where mutations in these genes do not affect their survival (Yip et al., 2007). The number of pseudogenes present in *M. ulcerans* and other ecological variants such as *M. liflandii* and *M. pseudoshottsii* varies as there are higher number of pseudogenes (771) present in *M. ulcerans* Agy99 whereas comparatively lower numbers of pseudogenes are present in *M. liflandii* (436) and *M. pseudoshottsii* (538), which suggests that *M. ulcerans* adapted to occupy more restrictive sites compared to other ecological variants (Doig et al., 2012; Tobias et al., 2013; Yoshida, Miyamoto, Ogur, Hayashi, & Hoshino, 2017). Moreover, there are some additional differences between *M. ulcerans* and other ecological variants such as *M. liflandii* and *M. pseudoshottsii* which include their plasmid size, host specificity, type of mycolactone produced and the absence of the MURD152 region in *M. ulcerans* (Nakanaga et al., 2013; Yip et al., 2007; Zingue et al., 2018). Mycolactone producing mycobacteria such as *M. liflandii* and *M. pseudoshottsii* have larger plasmid sizes and produce

mycolactone E and mycolactone F, respectively, and cause disease in ectotherms such as frog and fish, whereas *M. ulcerans* produces mycolactone A/B, C or D, leading to BUD pathology and human disease (Nakanaga et al., 2013; Yip et al., 2007; Zingue et al., 2018). In the evolutionary hierarchy, *M. liflandii* is suggested to be an intermediate between the ancestor *M. marinum* M and *M. ulcerans* Agy99, as *M. marinum* consists of all gene cluster present in *M. liflandii* and *M. liflandii* consists of all gene cluster present in *M. ulcerans* (Tobias et al., 2013). But there are some genes that are pseudogenized in *M. ulcerans* but not in *M. liflandii* and vice-versa indicating that there was an independent reductive evolution in significant part 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. In this study, we observed the effect of combined high temperature and low oxygen on upregulation of several stress response genes of *M. ulcerans*. It would be interesting to determine whether these effects are also observed for other MPMs. But these genes were similar to the stress response genes upregulated for *M. tuberculosis* on exposure to heat shock and hypoxic environment and during intracellular survival inside the macrophage. This suggest that *M. ulcerans* can modulate its gene expression to cope with environmental changesthat will enhance survival and provide a fitness advantage in its natural habitat and during infection of human hosts. Further, although mycolactone is considered as a major virulence factor, more investigations are needed to understand the impact of factors such as *clp*B that are known virulence determinants in other bacteria.

4.6 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 (Yip et al., 2007). In this study, *M. ulcerans* was exposed to nutritional and several other abiotic stresses to understand their effect on *M. ulcerans* growth, and mycolactone and global gene expression. Mycolactone expression was upregulated under microaerophilic conditions suggesting that *M. ulcerans* may reside in a microaerophilic habitat in the environment and mycolactone could provide a fitness advantage in those environments. The growth of *M. ulcerans* was not affected by exposure to UV for up to 10 mins, and there was a time-dependent downregulation of mycolactone on UV exposure although was statistically insignificant. This suggest that *M. ulcerans* may have higher tolerance to UV than previously thought and mycolactone may not play role to provide fitness advantage against it. The exposure of *M. ulcerans* to combined high temperature (37^0C) and low oxygen stress caused upregulation of several stress response genes at the expense of downregulation of mycolactone expression. 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 (O'Brien et al., 2014). Although, *M. ulcerans* was not able to utilize the bacterial quorum sensing molecule and insect hormone as a sole carbon source, more investigations with different concentrations and signaling molecules are needed to understand its ability to use chemical signals as a nutrient source, which is a mechanism for interfering or eaves dropping on quorum sensing signaling in other microbes (Leadbetter & Greenberg, 2000). The study on *M. ulcerans* growth and gene expression in response to these interspecies and interkingdom signaling can provide further insights on *M. ulcerans* ecology. There are some other 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. Additionally, investigations using a mycolactone negative mutant *M. ulcerans* and other MPMs are needed to further elucidate the role of mycolactone against these abiotic and biotic stresses. But altogether, these initial data increase our understanding of *M. ulcerans* response to a changing environment and may provide insight to pathogenesis upon host infection.

Table 4.1 List of differentially regulated genes, their names, up or down regulation, their product and KEGG/String functional categorization).

Name	**up/down**	**Location-Plasmid/chromosome**	**Product**	**KEGG/String/Uniprot-function annotation**
MUL_RS00170 (*mls*B)	down	Plasmid	Type I modular polyketide synthase	Metabolism/biosynthetic process
MUL_RS00210 (*mls*A2)	down	Plasmid	Type I modular polyketide synthase	Metabolism/biosynthetic process
rrf	up		Ribosome-recycling factor,chloroplastic (Organism: wild carrot)	Genetic information processing/translational termination
*clp*B	up	Chromosome	Chaperone protein ClpB	Genetic information processing/Stress response
MUL_RS04235 (MUL_0753)	up	Chromosome	Acyl-CoA dehydrogenase	Metabolism
MUL_RS05050 (MUL_0911)	up	Chromosome	Integral membrane transport protein	Environmental information processing/Transport
MUL_RS08325 (MUL_1522)	up	Chromosome	Uncharacterized protein	Cytoplasmic protein

Table 4.1 (continued)

Name	**up/down**	**Location-Plasmid/chromosome**	**Product**	**KEGG/String/Uniprot - function annotation**
MUL_RS25225 (MUL_4884)	up	Chromosome	Dioxygenase	Metabolism/cellular metabolic process
MUL_RS04920 (*gro*S/*gro*ES)	up	Chromosome	10 kDa chaperonin	Genetic information processing/ Stress response
MUL_RS07010 (*acc*D6)	up	Chromosome	Acetyl/propionyl-CoA carboxylase (Beta subunit) AccD6	Metabolism
MUL_RS03425 (*dna*K)	up	Chromosome	Chaperone protein DnaK	Genetic Information Processing, Human Diseases, signaling and cellular processes/Stress response
*gro*l_1 *(groL1*/ *gro*EL1)	up	Chromosome	60 kDa chaperonin 1	Genetic Information Processing, Human Diseases, signaling and cellular processes/protein refolding

Table 4.1 (continued)

Name	**up/down**	**Location-Plasmid/chromosome**	**Product**	**KEGG/String/Uniprot-function annotation**
*gro*l_2 (*gro*L2/ *gro*EL2)	up	Chromosome	60 kDa chaperonin 2	Genetic Information Processing, Human Diseases, signaling and cellular processes/protein refolding
MUL_RS20535	Up	Chromosome	-	-
MUL_RS01465 (MUL_0208)	up	Chromosome	Conserved hypothetical transmembrane protein	Conserved hypothetical transmembrane protein/ Cellular component
MUL_RS17465 (*sig*B)	up	Chromosome	RNA polymerase sigma factor	Genetic information processing/Transcription , Transcription regulation
MUL_RS04190 (MUL_0744)	up	Chromosome	Dioxygenase	Metabolism/ metal ion binding/oxidoreductase activity
*mms*A	up	Chromosome	Methylmalonate semialdehyde dehydrogenase, MmsA	Metabolism/(methylmalonate-semialdehyde dehydrogenase (acylating) activity
MUL_RS06415 (*hsp*20)	up	Chromosome	Heat shock protein Hsp	Environmental information processing/stress response

Table 4.1 (continued)

Name	**up/down**	**Location-Plasmid/chromosome**	**Product**	**KEGG/String/Uniprot-function annotation**
MUL_RS00610 (*whi*B5)	up	Chromosome	Transcriptional regulator WhiB	Genetic information processing/Transcription, Transcription regulation
MUL_RS10665 (*mas*)	up	Chromosome	Multifunctional mycocerosic acid synthase membrane-associated Mas	Metabolism/ oxidoreductase activity
MUL_RS21985 (MUL_4235)	down		Uncharacterized protein	Cytoplasmic protein
MUL_RS25940 (*eth*A)	up	Chromosome	Monooxygenase EthA	Metabolism/Monooxygenase Oxidoreductase
MUL_RS15640 (*ahp*D)	up	Chromosome	Alkyl hydroperoxide reductase AhpD	Metabolism/response to oxidative stress
MUL_RS20530	up	Chromosome	23S RNA	

CHAPTER V

IMPACT OF MYCOLACTONE IN POLYMICROBIAL INTERACTIONS OF MYCOBACTERIUM ULCERANS IN AQUATIC ENVIRONMENTS

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5.1 Abstract

Mycolactone is a cytotoxic lipid toxin produced by an environmental pathogen *Mycobacterium ulcerans.* The pathogen causes Buruli ulcer disease (BUD), a necrotizing skin lesion. Mycolactone is mainly responsible for disease pathology of BUD. Plasmid pMUM001is responsible for mycolactone synthesis and was acquired during evolution from its progenitor *M. marinum* (Yip et al., 2007). Acquisition of this huge plasmid and its maintenance suggests its specific role to provide a fitness advantage in the environment. Further, mycolactone is structurally similar to acyl homoserine lactone (AHL) (a quorum sensing molecule) and *M. ulcerans* can form biofilms (Marsollier et al., 2004). Additionally, continuous increases in mycolactone concentration was observed even after bacterial cultivation reached a plateau in an animal model which suggested that mycolactone production may be population-density dependent and be involved in a quorum sensing mechanism (Fred Stephen Sarfo et al., 2013). Quorum sensing signals of one bacterium (such as *Pseudomonas aeruginosa*) has been shown to quench the quorum sensing mechanism of other bacteria (*Staphylococcus aureus*) (Qazi et al.,

2006). Hence, this study was focused on investigating the effect of mycolactone in determining the microbial community in its vicinity. For this pilot study, mycolactone coated and uncoated slides were exposed to the aquatic habitat in Buruli ulcer disease endemic and non-endemic locations. The relative abundance, most important bacterial taxa, richness and diversity were determined based on endemicity and treatment. There was no significant effect of mycolactone on richness and diversity. However, based on random forest modeling, family Comamonadaceae was the best predictor for both *M. ulcerans* endemicity and mycolactone treatment as there was higher abundance of these in endemic locations than in non-endemic locations on control slides; but also, mycolactone addition to glass slides showed increased abundance of Comamonadaceae compared to controls, no matter the location. On the other hand, Sphingomonadaceae and Intrasporangiaceae were lower in abundance in endemic locations compared to non-endemic locations from control slides, and mycolactone addition reduced their abundance further in both endemic and non-endemic sites. Interestingly, though Bradyrhizobiaceae was an important predictor for endemicity, as it had higher abundance in endemic locations compared to the non-endemic location, mycolactone addition reduced its abundance, no matter the location. These families belonged to both gram positive and gram-negative bacteria. They were mostly mesophilic, aerobic or facultatively aerobic and were motile or non-motile. There was no significant difference (chi-square test) in general characteristics such as gram status, temperature requirement, oxygen tolerance and motility of these top 10 most important taxa based on their affinity to mycolactone. However, when these top 10 most important taxa were grouped based on phylum there was a positive relationship of mycolactone with Firmicutes and negative relationship with Actinobacteria. Among Proteobacteria, gamma-proteobacteria had a positive relationship with mycolactone whereas alpha- proteobacteria had negative or variable and beta-

proteobacteria had positive or variable relationship with mycolactone. Hence, although there was no clear differentiation of microbial community based on their attachment to mycolactone, the difference in phylum level affinity to mycolactone for the top 10 most important bacterial taxa suggests its impact on determination of microbial community in its vicinity.

5.2 Introduction

Environmental pathogens normally reside in the environment outside of a host, but can infect humans following incidental contact or through known and demonstrated vectors (American Society For Microbiology, n.d.). During their lifecycle within the environment, these pathogens encounter many complex biotic (such as competing microbes, host plants and invertebrates) and abiotic (such as salinity, temperature, oxygen concentration) factors that influence their persistence in the environment. The stress caused by these abiotic and biotic factors can either cause death of the organism or may induce adaptation (Perincherry et al., 2019). Hence, these pathogens acquire traits such as adhesion and secondary metabolites for nutrient acquisition and competition during their adaptation in the environment that can contribute to virulence upon infection of a human host (Sakib et al., 2018; S. Sun et al., 2018). The structure and function of these naturally produced compounds are quite diverse, but the widespread production in natural environments, together with evolutionarily conserved genes, suggests that they have important functions for the survival of the producers (Sharrar et al., 2020). Thus, the primary functions of these traits in an environmental pathogen are to facilitate their environmental lifestyle rather than enhance pathogenicity in humans.

Cytotoxic compounds are one of the secondary metabolites that are produced in response to external stress (Perincherry et al., 2019). It has been suggested that several virulence factors such as toxins, Type VI Secretion Systems, quorum sensing, N-acetylglucosamine binding

protein, toxin-coregulated pilus, and outer membrane proteins present in environmental pathogens such as *Vibrio cholerae* provide an environmental advantage by protecting against predators, facilitating colonization and interactions with the host, conferring a defensive role and competitive edge and enhancing biofilm formation, nutrient acquisition and genetic exchange (Sakib et al., 2018). Similarly, cereulide produced by an environmental pathogen *Bacillus cereus* with emetic toxin activity in humans, aids bacterial survival in its natural environment by scavenging potassium from soil, enhancing symbiotic relationships with host plants and competing against other bacteria and fungi (Ehling-Schulz et al., 2015). In fungi, mycotoxins are produced in response to stress caused by various biotic (e.g. bioactive plant metabolites) and abiotic determinants (e.g. pH and temperature) (Perincherry et al., 2019).

In their natural habitats, environmental pathogens sustain symbiotic relationships with other microbes, invertebrates, plants, amphibians and reptiles, among others. Their patterns of co-existence manifest as synergistic or antagonistic and are regulated by biotic and abiotic interactions where species diversity and environmental conditions are the driving determinants. For example, mycotoxins produced by *Fusarium* spp such as fusaric acid, zearalenone and fumonisin aid in facilitating antagonistic relationships against bacteria through quorum quenching activity, thereby protecting *Fusarium* against antimicrobials (Venkatesh & Keller, 2019). On the other hand, other mycotoxins produced by *Fusarium* spp such as deoxynivalenol provide synergistic relationships to microbes during infection by reducing immunity in a susceptible host and enhancing other mechanisms of microbial diseases such as Coccidiosis (Antonissen et al., 2014). Further, toxins produced by *Fusarium* spp can have both synergistic and antagonistic relationships with a pathogen as it has been shown that deoxynivalenol directly enhances virulence of *Salmonella* Typhimurium by increasing the expression of the *Salmonella*

pathogenicity island (SPI) that aid in invasion. Yet, the *Fusarium* T-2 toxin decreases motility and expression of SPI thereby decreasing virulence (Antonissen et al., 2014).

It is also assumed that toxic secondary metabolites provide protection to microbes by cleansing the immediate environment of predators and competing microbes (Jousset, 2012). For example, toxic secondary metabolites produced by *V. cholerae* and *Pseudomonas fluorescens* protect each against nematode and protozoan predators (Jousset, 2012). Additionally, these products defend neighboring microbes against predators, thereby influencing microbial community composition within the local area (Jousset, 2012).

But despite the examples above and a few others, in many cases, the benefit these compounds confer on the organism is unknown. Therefore, understanding the biotic and environmental factors that influence the production of compounds that later come to be virulence factors upon human infection, is important for examining and predicting microbial relationships, adaptation, and transmission. Also, communities are interacting networks of species that change in space and time. Identifying mechanisms into how communities assemble and change has been a key tenet of community ecology (Jackson & Blois, 2015; Stroud et al., 2015), but also has direct application in understanding how microbes evolve into pathogenic organisms (Percy, 2007).

We conducted a pilot study to investigate the impact of mycolactone, a cytotoxic lipid compound secreted by the environmental pathogen *Mycobacterium ulcerans,* on determining microbial community composition. *Mycobacterium ulcerans* causes Buruli ulcer disease, a necrotizing skin disease with high morbidity in affected individuals and families. Mycolactone is the major virulence determinant that is responsible for the tissue necrosis and painlessness pathology observed in Buruli ulcer wounds(Marion, Song, et al., 2014; Fred Stephen Sarfo et al.,

2016). The painlessness is mainly due to immunosuppression via blockage of sec61 pathway and cytoskeleton hyperactivation. Mycolactone is encoded on a giant plasmid pMUM001, that was likely acquired by *M. ulcerans* during evolution from its *M. marinum* progenitor(Yip et al., 2007). Buruli ulcer prevalence is associated with contact with aquatic environments, which has been strengthened by *M. ulcerans* DNA detected from aquatic plants, soil, vertebrates and invertebrates (J. A M Fyfe et al., 2007; Vandelannoote et al., 2010; Williamson et al., 2012). *M. ulcerans* can also survive intracellularly inside amoebae (Amissah et al., 2014; Azumah et al., 2017; Gryseels et al., 2012) and host macrophages (Torrado et al., 2007), however, they are found as primarily extracellular in patient tissues. And though wild type *M. ulcerans* has been shown to have an initial phase of intracellular proliferation during early infection, cell lysis occurs with sufficient bacterial numbers (Torrado et al., 2007). And mycolactone-negative mutants maintain intracellular survival (Adusumilli et al., 2005; Torrado et al., 2007). This intracellular replication followed by cell lysis and extracellular proliferation of wild type *M. ulcerans* could also reflect its lifestyle in its natural habitat as a means of proliferation and dispersal. Furthermore, mycolactone consists of a 12-membered lactone ring with acyl side chains and is structurally similar to AHL, a quorum sensing molecule in many gram-negative bacteria. This structural similarity of mycolactone suggests that mycolactone could have quorum sensing or quenching properties, that could influence cell-to-cell communication and phenotypes of other microbes or defending itself and other neighboring microbes against predation. Thus, despite being a major virulence factor of human disease, the primary, evolved, target of mycolactone in natural environments remains unknown, but its production is likely providing a fitness advantage in the environment that shapes community structure and functioning.

5.3 Materials and Methods

5.3.1 Sites used for the study

Both *M. ulcerans* endemic and non-endemic aquatic habitats were selected from Tchaihoue and Ganlohoue, two hamlets in the village of Tanji, in the commune of Lalo, Benin. Samples were collected from these locations in May of 2013 and 2014 (Figure 5.1A). Within Tchaihoue, samples were collected from the men's bathing area (Figure 5.1B) and ladies bathing area (Figure 5.1C). Within Ganlohoue, samples were collected from the rice field.

5.3.2 Experimental design

Mycolactone (500 ng in a 10 µl ethanol volume) was bound to slides by pipetting directly to slides, spreading to cover the slide surface and allowing the slides to air dry. Mycolactone's hydrophobicity allows the compound to adhere to slides during exposure to water. Mycolactone-bound or control (coated with 10µL ethanol and allowed to dry) glass slides (N=4/chamber) were placed inside plexiglass field microcosm chambers covered with 0.2µm nitrocellulose membranes (Figure 5.1D). Fifty mL water from the respective aquatic habitat was loaded into replicate mesocosm chambers (N=4) through the syringe needle, that was then sealed with parafilm and duct tape. Chambers were then surrounded by mesh and immersed within the respective aquatic habitat in a location with signs of minimal human or animal disturbance for 10 days. These chambers with appropriate pore size filters allow nutrients to pass through via diffusion, while excluding entry of external organisms or exit of most internal microbes loaded into the chamber from the water sample. Aquatic habitats were chosen based on previous *M. ulcerans* positivity within each site. The Tchaihoue ladies bathing area and Ganlohoue rice field were considered endemic for *M. ulcerans*, as previous sampling over a six year period yielded more than three matrices positive with an average concentration of 1,000 genome units per

sample (data not shown). The men's bathing area was considered non-endemic for *M. ulcerans* based on previous sampling over a six year period showing one or fewer matrices positive, with fewer than 100 genome units per sample (data not shown). Control and treatment glass slides were harvested and preserved with RNA Later. Slides were scraped or sonicated to remove the biofilm, for DNA isolation and subsequent targeted 16s metagenomic sequencing.

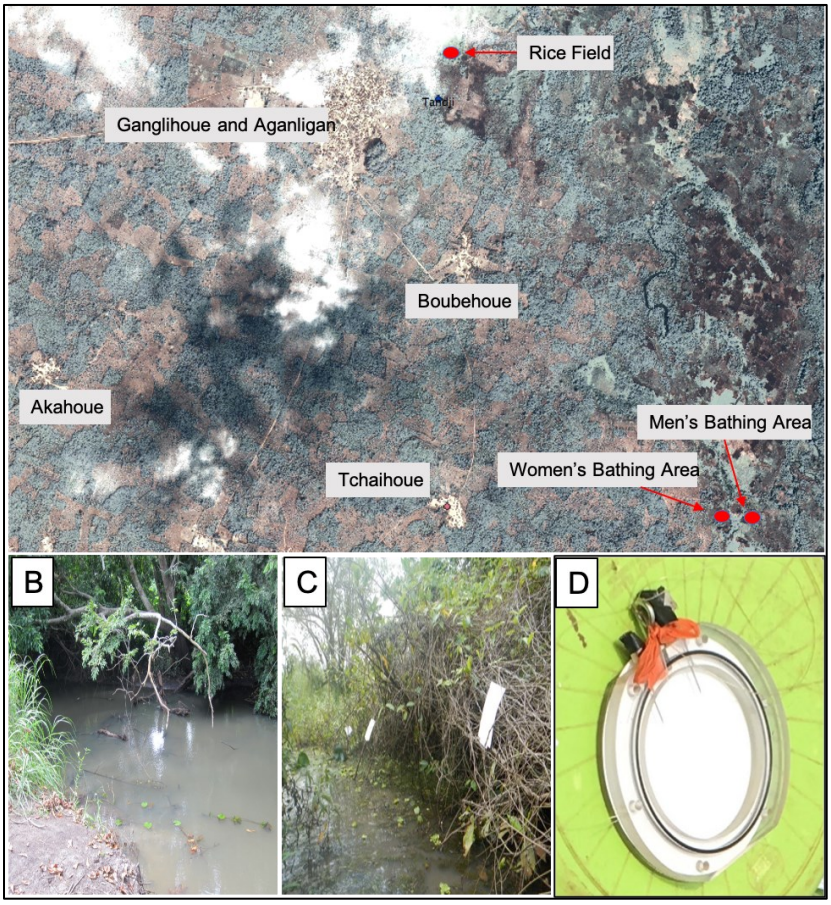

Figure 5.1 Locations and chamber used for study.

A) aerial view of locations sampled in Tanji village. Red dots indicate specific aquatic habitats used for the study. B) Photo of the Tchaihoue men's bathing area, and c) Tchaihoue ladies' bathing area, two aquatic habitats used for the study, D) Photo of mesocosm chamber. The photo shows the bottom portion covered with a 0.2 □m nitrocellulose filter, with the front portion open where slides will be enclosed and also covered with a 0.2 □m nitrocellulose filter. Fifty mL water from the aquatic habitat is added through one of the syringe needles at the top, then sealed with parafilm and duct tape.

5.3.3 DNA extraction and 16S sequence processing

DNA was extracted using the protocol adapted from Lamour and Finley (Lamour & Finley, 2006) and discussed by Williamson *et al*. (2008) (Williamson et al., 2008). Briefly, environmental samples were homogenized in presence of lysis solution (500 µl) (100 mM Tris (pH8.0), 50 mM EDTA, 500 mM NaCl, 1.33% SDS and 0.2 mg/mL RNase A) and glass beads (0.5g of a mixture of 0.2 and 0.5 micron). Samples were incubated at 65^0C for 20 minutes and centrifuged. One hundred fifty microliters of potassium acetate (5 M) was added to the supernatant and allowed to chill overnight at -20^0C. Following 30 minutes centrifugation at 4^0C, supernatants were added to 1.6 mL guanidium hydrochloride (0.66 M guanidine hydrochloride in 63.3% ethanol solution). The mixture was transferred to a MOBIO spin filter and centrifuged at 5600g for 2 minutes. The flow through was discarded and the procedure was repeated until all sample was ran through the spin filter. The spin filter was washed with wash solution (500 µl) (10 mM Tris [pH 8], 1 mM EDTA, 50 mM NaCl, 67% ethanol) followed by ethanol (500 µl). Final sample DNA was eluted using elution solution (200 µl) and quantified using qubit 2.0. DNA from triplicate sample replicates were combined for 16S sequence analysis.

DNA was sent to Michigan State Research Technology Support for 16s sequencing of 2 x 300 bp paired-end reads. 16S library construction and sequencing was performed using a modified version of the protocol adapted for the Illumina HiSeq2000 as described by Caporaso and Bittinger (Caporaso, Bittinger, et al., 2010). Briefly, the V4 region of the 16S rRNA gene was amplified with region-specific primers that included the Illumina flowcell adapter sequences. The final libraries were normalized and pooled using a PicoGreen assay and subsequently quantified using the Kappa qPCR kit. After cluster formation on the HiSeq instrument the amplicons were sequenced with custom primers. These sequencing primers were

designed to be complimentary to the V4 amplification primers to avoid sequencing of the primers, and the barcode is read using a third sequencing primer in an additional cycle. The amplification primers were adapted from the Caporaso, Kuczynski (Caporaso, Kuczynski, et al., 2010) protocol to include nine extra bases in the adapter region of the forward amplification primer that support paired-end sequencing on the HiSeq (Caporaso, Kuczynski, et al., 2010). Mock-communities of known assemblages was included for each sequencing run to account for run-to-run sequencing variability (Caporaso et al., 2012). Furthermore, the filtering parameters for sequence classification was optimized using the setting recommended by Caporaso, Lauber (Berg-Lyons et al., 2010) for samples, as phylogenetic diversity among OTUs with low abundance can be affected by the filtering parameters (Caporaso et al., 2012).

5.3.4 Sequence analysis and modeling

Sequences were quality filtered then ran through the most up- to-date GreenGenes database (Second Genome Inc, n.d.). Sequences were then analyzed for relative abundance using phyloseq in the R package (Kaszubinski & Receveur, n.d.). Taxon abundance was shown for taxa >2%. Taxa <2% were removed from each sample for determining relative abundance and determining the top 10 most important taxa (random forest). Relative abundance was calculated based on treatment and endemicity. Total OTU percentage was calculated based on endemicity and treatment. For determining total OTU based on endemicity, samples from locations with the same endemicity were combined. Similarly, samples from the same treatment group were combined separately in excel to determine total OTU percentage based on treatment. Statistical analysis of bacterial community change followed previously described methods (Pechal et al., 2014, 2013). Shannon indices were determined for each sample, then compared using the Wilcoxson rank sum test was used to determine the significant differences. Taxon turnover was

calculated according to Whittaker's beta diversity (Whittaker, 1960). Beta diversity measures taxon turnover rates or changes in communities along spatial or temporal gradients or among treatment groups (Scheiner, 1992; Whittaker, 1960). The significant difference in beta diversity was determined based on Permutational Analysis of Variance (PERMANOVA) using distance matrix ("bray"). Function Adonis, which is a PERMANOVA test based on dissimilarities was performed using vegan version 2.5-6 library in R to test significant difference in beta diversity between endemic and non-endemic locations, and between control and mycolactone treated groups across all locations, and in endemic and non-endemic locations (permutations =999). There was no significant difference in beta diversity for control slides (P=0.8) and endemic locations (P=0.5) by year. Because of these results, we analyzed all data within a single cohort. DNA from the men's bathing did not amplify for 2014 samples and were thus not included in the above analyses.

We used random forest classification to identify taxa that may be differentially abundant in the endemic versus nonandic locations, or among control samples compared with those treated with mycolactone. For this, the top 10 most important taxa for classifying were determined using Random Forest v 4.6-14 package in R(Kaszubinski & Receveur, n.d.). The random forest algorithm generates different numbers of trees for a data frame to make a decision based on the input of each individual tree (Breiman, 2001; X. Chen & Ishwaran, 2012; J. Thompson, Johansen, Dunbar, & Munsky, 2019). The outcome determines important taxa by bootstrap method based on their prediction for a variable when included in the decision tree. The random forest algorithm determined important operational taxonomic units (OTUs) for treatment and locations, which were then sorted in descending order based on their MeanDecreaseGini (X. Chen & Ishwaran, 2012; J. Thompson et al., 2019).

Further, the top 10 most important taxa were classified as having synergistic, antagonistic or variable relationships with mycolactone based on the increase or decrease in % OTU abundance in mycolactone treated slides in both endemic and non-endemic locations. The chi-square test was performed to determine the significant difference in general characteristics (such as gram status, temperature and oxygen requirement and motility) of these families based on their affinity to mycolactone.

5.4 Results

5.4.1 Relative abundance, species richness and most important taxa based on locations

Three different sites in Tanji village were selected based on *M. ulcerans* endemicity. The ladies bathing area and rice field were endemic for *M. ulcerans* whereas the men's bathing area is non-endemic, based on six years of *M. ulcerans* sampling data. Microbial DNA from control slides (without mycolactone) from their respective mesocosm chambers were used for this analysis, in order to determine natural microbial community composition based on *M. ulcerans* endemicity. The relative bacterial abundance above 2%, based on endemicity is shown in Figure 5.2. Higher species richness (Chao1) and evenness (Shannon) was observed for endemic locations compared to the non-endemic location. However, there was no significant difference in Shannon diversity (P= 0.37) in endemic and non-endemic locations (Figure 5.3). The random forest algorithm determined the top ten most important taxa with relative abundance above 2% for classifying *M. ulcerans* endemicity (Table 5.1). They were Caulobacteraceae, Bradyrhizobiaceae, Chitinophagaceae, Nocardioidaceae, Burkholderiaceae, Xanthomonadaceae, Clostridiaceae, Micrococcaceae, Bacillaceae and Comamonadaceae. The percentage based on total OTUs for each of these taxa according to endemicity showed higher abundance of family Caulobacteraceae (13.23%), Bradyrhizobiaceae(3.68%), Chitinophagaceae(0.54%),

Burkholderiaceae(0.97%), Xanthomonadaceae(1.37%), Clostridiaceae (0.34%), Bacillaceae (2.6%) and Comamonadaceae (5.06%) in endemic aquatic habitats, whereas higher percentage abundance of Nocardioidaceae (16.71%) and Micrococcaceae (17.92%) was observed in the non-endemic location (Table 5.1).

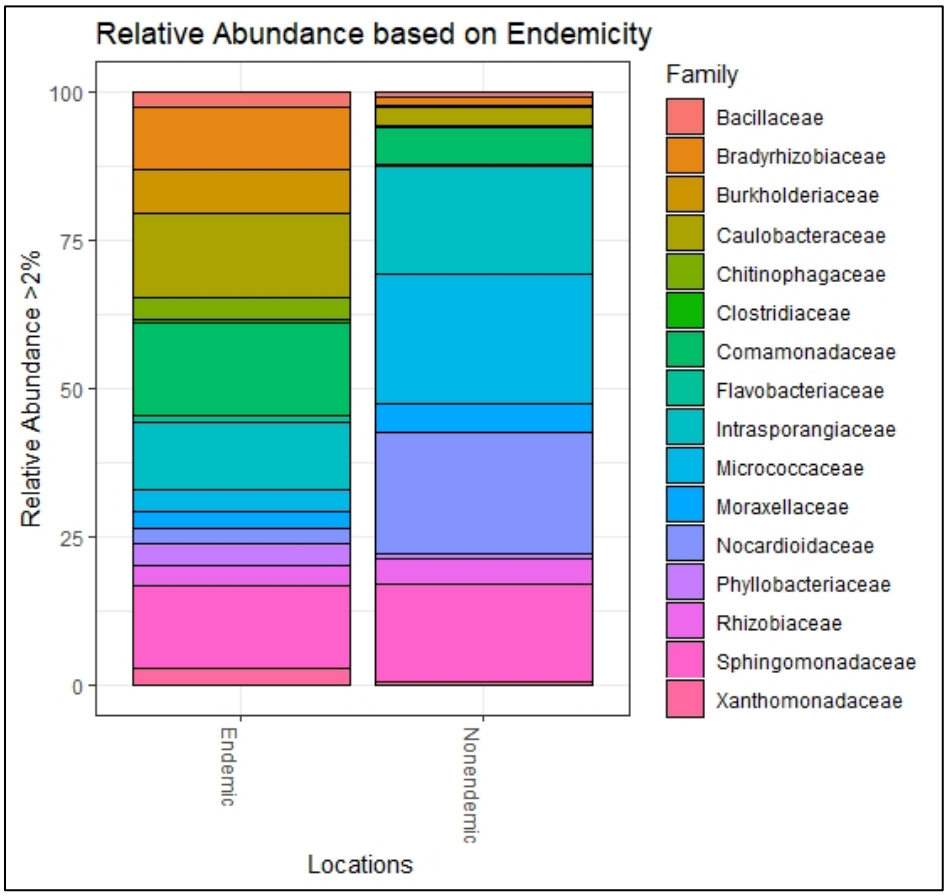

Figure 5.2 Relative abundance (>2%) based on locations.

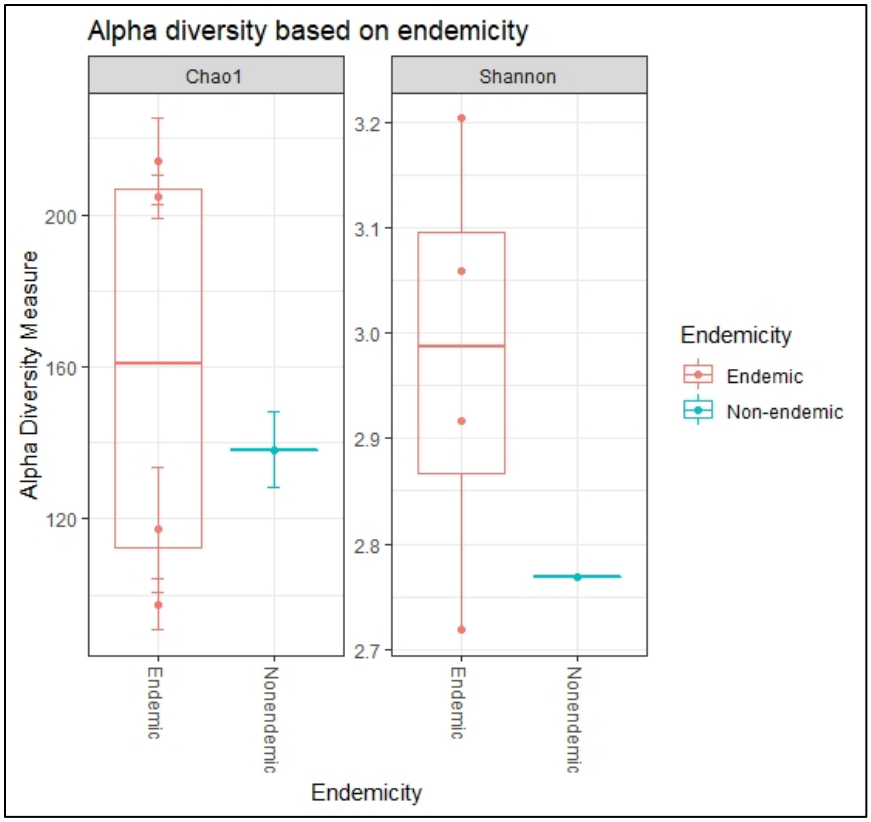

Figure 5.3 Alpha diversity (Chao1 and Shannon diversity) based on locations

5.4.2 Relative abundance, species richness and most important taxa based on mycolactone treatment

The relative abundance for mycolactone coated versus uncoated slides from all aquatic locations were compared and the most important taxa were determined using the random forest algorithm. The relative abundance (>2%) is shown in Figure 5.4. Higher species richness (chao 1) and evenness (Shannon) was also observed for untreated slides compared to mycolactone treated slides. However, there was no significant difference in Shannon diversity (P=0.44) and in beta-diversity based on Adonis analysis (P=0.753, permutations= 999) between control and mycolactone treated slides from all aquatic locations (Figure 5.5). The top 10 most important taxa for classifying treatment among species with relative abundance above 2% was determined,

and showed that Nocardioidaceae, Sphingomonadaceae, Caulobacteraceae, Burkholderiaceae, Flavobacteriaceae, Bradyrhizobiaceae, Intrasporangiaceae, Comamonadaceae, Phyllobacteriaceae and Chitinophagaceae were the most important taxa based on treatment from all aquatic locations (Table 5.1). The higher percentage of total OTUs was observed for Nocardioidaceae (3.72%), Burkholderiaceae (1.042%), Flavobacteriaceae (1.56%), Comamonadaceae (11.60%), Phyllobacteriaceae (1.32%) and Chitinophagaceae (0.64%) in mycolactone coated slides whereas higher percentage of total OTUs was observed for taxa Sphingomonadaceae (6.07%), Caulobacteraceae (12.93%), Bradyrhizobiaceae (3.61%) and Intrasporangiaceae (11.72%) for control slides from all aquatic locations (Table 5.1).

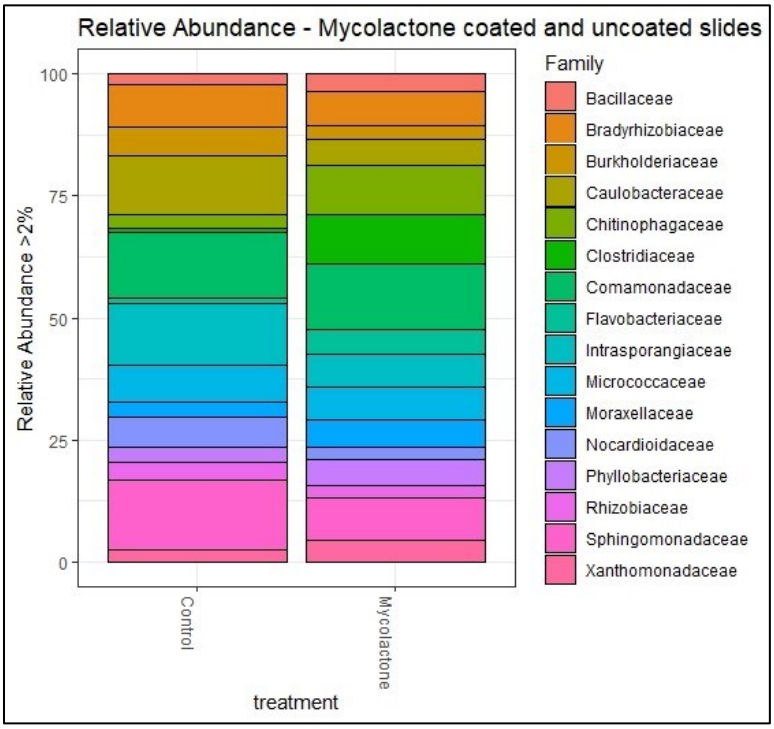

Figure 5.4 Relative abundance (>2%) based on treatment

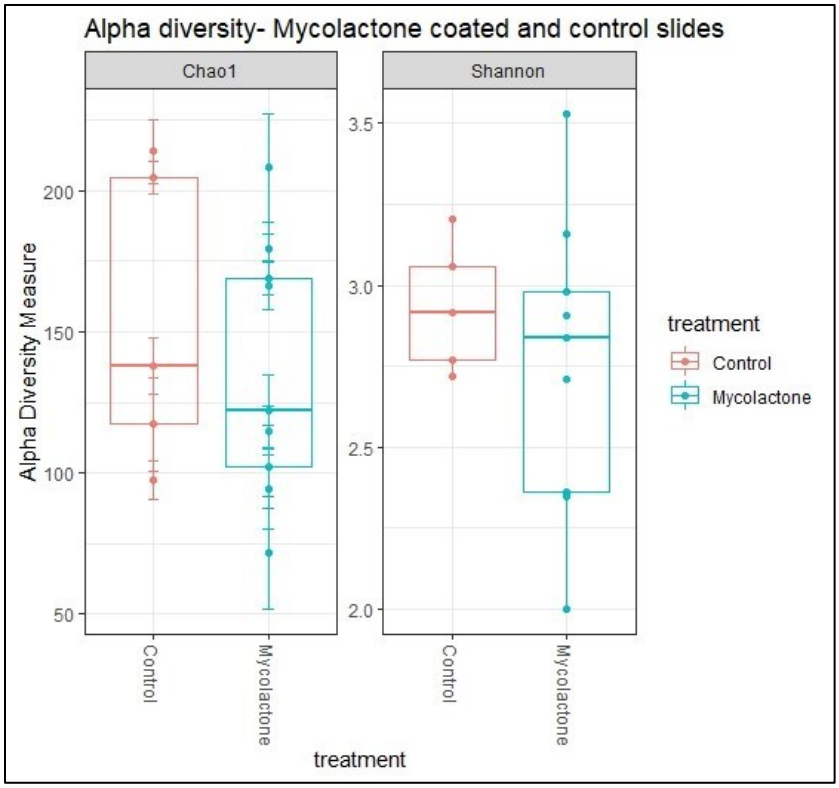

Figure 5.5 Alpha diversity (Chao1 and Shannon diversity) based on treatment

5.4.3 Relative abundance, species richness and most important taxa based on locations and treatment

The relative abundance and most important taxa were determined based on treatment and compared across *M. ulcerans* endemicity. The relative abundance (>2%) based on treatment for *M. ulcerans* endemic locations is shown in Figure 5.6. In endemic locations, the top ten most important taxa for relative abundance above 2% were Caulobacteraceae, Nocardioidaceae, Burkholderiaceae, Chitinophagaceae, Flavobacteriaceae, Bradyrhizobiaceae, Moraxellaceae, Intrasporangiaceae, Comamonadaceae and Xanthomonadaceae based on treatment (Table 5.2). Among them, higher percentage OTUs were measured for Burkholderiaceae (1.83%), Chitinophagaceae (1.11%), Flavobacteriaceae (2.2%), Moraxellaceae (6.36%),

Comamonadaceae (10.43%) and Xanthomonadaceae (1.67%) in mycolactone treated slides compared to control slides in endemic location. But, higher percentage OTUs were measured for Caulobacteraceae (13.24%), Nocardioidaceae (2.71%), Bradyrhizobiaceae (3.68%) and Intrasporangiaceae (11.62%) in control slides compared to mycolactone treated slides in endemic location (Table 5.2). In *M. ulcerans* endemic locations, higher species richness and evenness was observed for untreated slides compared to mycolactone treated slide but there was no significant difference in Shannon diversity (P=0.93) and in beta diversity (P=0.62, permutations =999) between the treatment groups (Figure 5.7).

Similarly, the top 10 most important taxa for relative abundance above 2% in *M. ulcerans* non-endemic locations based on treatment were Sphingomonadaceae, Comamonadaceae, Chitinophagaceae, Clostridiaceae, Caulobacteraceae, Bradyrhizobiaceae, Bacillaceae, Nocardioidaceae, Phyllobacteriaceae and Micrococcaceae (relative abundance >2% shown in Figure 5.6 and Table 5.2). Among these taxa, higher percent OTUs were observed for Comamonadaceae (13.02%), Clostridiaceae (0.49%), Caulobacteraceae (8.49%) and Bacillaceae (3.97%) in mycolactone coated slides compared to control slides whereas higher percent OTUs were observed for Sphingomonadaceae (13.52%), Chitinophagaceae (0.19%), Bradyrhizobiaceae (1.05%), Nocardioidaceae (16.71%), Phyllobacteriaceae (0.6%) and Micrococcaceae (17.92%) in control slide compared to mycolactone coated slides in non-endemic location(Table 5.2). For non-endemic locations, mycolactone treated slides had higher species richness (chao 1) but the evenness was lower based on the Shannon index. However, there was no significant difference in Shannon diversity (P=0.67) in these treatment groups in non-endemic locations (Figure 5.8).

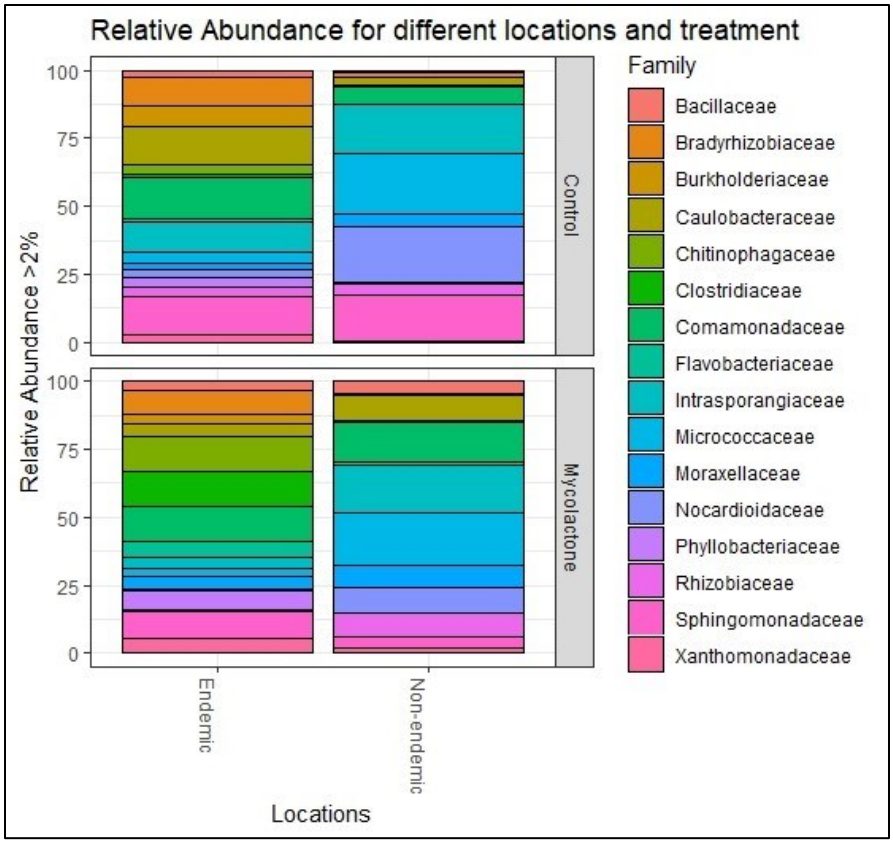

Figure 5.6 Relative abundance (>2%) based on treatment for *M. ulcerans* endemic and non-endemic locations.

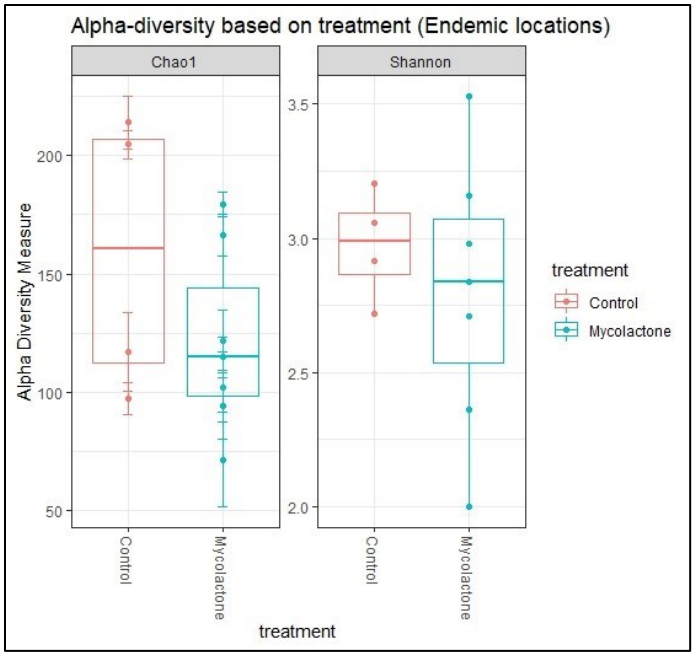

Figure 5.7 Alpha diversity (Chao1 and Shannon diversity) based on treatment for *M. ulcerans* endemic locations

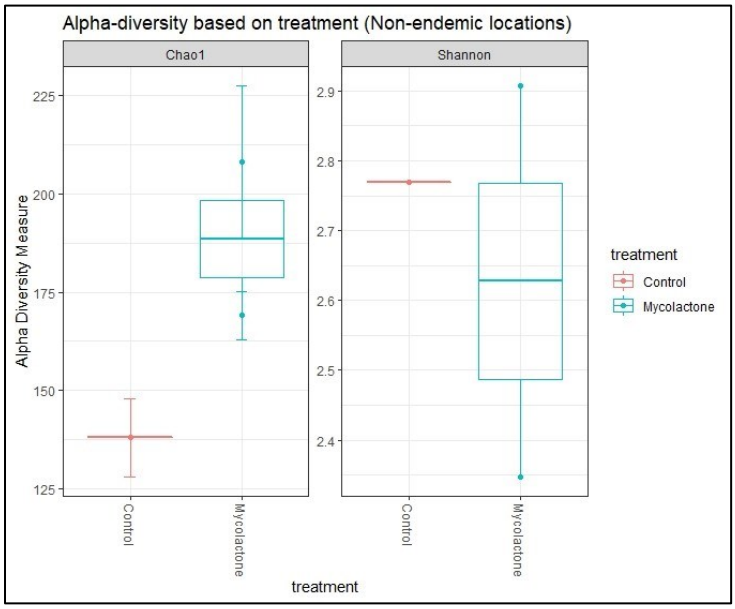

Figure 5.8 Alpha diversity (Chao 1 and Shannon diversity) based on treatment for the *M. ulcerans* non-endemic location

Table 5.1 Top 10 most important taxa for classifying *M. ulcerans* endemicity and treatment

Top 10 most important taxa based on locations (M. ulcerans Endemic vs Non-endemic)			
Most important taxa	Endemic (%OTU)	Non-endemic (%OTU)	Increase/Decrease (Endemicity vs. Non-endemicity)
Caulobacteraceae	13.23673	2.559638943	Increase
Bradyrhizobiaceae	3.684701	1.050934881	Increase
Chitinophagaceae	0.545369	0.193423598	Increase
Nocardioidaceae	2.709153	16.71179884	Decrease
Burkholderiaceae	0.975356	0.161186331	Increase
Xanthomonadaceae	1.367268	0.509348807	Increase
Clostridiaceae	0.343644	0.116054159	Increase
Micrococcaceae	3.004336	17.9174726	Decrease
Bacillaceae	2.596079	0.85106383	Increase
Comamonadaceae	5.062354	5.016118633	Increase
Top 10 most important taxa based on Treatment (Mycolactone vs Control)			
Most important taxa	Control (%OTU)	Mycolactone (%OTU)	Increase/decrease (Mycolactone vs. Control)
Nocardioidaceae	3.114700528	3.7272586	Increase
Sphingomonadaceae	6.07796088	3.747517943	Decrease
Caulobacteraceae	12.92750105	4.47305565	Decrease
Burkholderiaceae	0.951776294	1.042780595	Increase
Flavobacteriaceae	0.358713412	1.558357842	Increase
Bradyrhizobiaceae	3.608421642	2.145418338	Decrease
Intrasporangiaceae	11.71822044	10.01237431	Decrease
Comamonadaceae	5.061014892	11.60411403	Increase
Phyllobacteriaceae	0.790626021	1.318699028	Increase
Chitinophagaceae	0.535175762	0.637593743	Increase

Table 5.2 Top 10 most important taxa based on treatment in *M. ulcerans* endemic and non-endemic locations

Top 10 most important taxa based on Treatment (Endemic locations)			
Most important taxa	Control (%OTU)	Mycolactone(%OTU)	Increase/Decrease (Mycolactone vs. Control)
Caulobacteraceae	13.23673356	1.142176073	Decrease
Nocardioidaceae	2.709152621	0.827361687	Decrease
Burkholderiaceae	0.97535648	1.831398471	Increase
Chitinophagaceae	0.545368884	1.114169174	Increase
Flavobacteriaceae	0.361720335	2.186643868	Increase
Bradyrhizobiaceae	3.684701403	3.63858045	Decrease
Moraxellaceae	2.591079103	6.357776547	Increase
Intrasporangiaceae	11.62428007	7.19377194	Decrease
Comamonadaceae	5.062353971	10.42719996	Increase
Xanthomonadaceae	1.367268252	1.666936909	Increase
Top 10 most important taxa based on treatment (Non-endemic locations)			
Most important taxa	Control (%OTU)	Mycolactone (%OTU)	Increase/decrease (Mycolactone vs. Control)
Sphingomonadaceae	13.52675693	3.104719539	Decrease
Comamonadaceae	5.016118633	13.02316606	Increase
Chitinophagaceae	0.193423598	0.062967815	Decrease
Clostridiaceae	0.116054159	0.485969349	Increase
Caulobacteraceae	2.559638943	8.489229457	Increase
Bradyrhizobiaceae	1.050934881	0.345053472	Decrease
Bacillaceae	0.85106383	3.974843342	Increase
Nocardioidaceae	16.71179884	7.223779491	Decrease
Phyllobacteriaceae	0.606060606	0.267867118	Decrease
Micrococcaceae	17.9174726	14.93987589	Decrease

5.5 Discussion

This study was performed to understand the role of mycolactone in determining polymicrobial interactions of *M. ulcerans* with other bacteria in its natural aquatic habitat. Control slides and slides treated with mycolactone were placed in endemic habitats and a non-endemic habitat to allow attachment of microbes. This was based on the hypothesis that mycolactone interacts selectively with microbes that would either attract some microbial species and repel other species. In this context, mycolactone could be acting as a signaling molecule, impact functional dynamics or exert individual effects on members of microbial community to drive this selective interaction. Further, sampling in *M. ulcerans* endemic and non-endemic provide further insights on the role of external environment in determining mycolactone interactions.

We found that alpha diversity (Chao 1 and Shannon diversity) was higher for endemic locations. The endemic locations are organically rich with high plant and insect communities that could influence the abundance of overall microbial community structure. Whereas, the non-endemic location is faster flowing, with very little plant and invertebrate diversity, based on our sampling. Application of mycolactone to slides lowered the alpha diversity in endemic locations, however, higher Chao 1 but lower Shannon diversity was observed for mycolactone coated slides in the non-endemic location. But, the difference in alpha diversity was not statistically significant, nor were differences in beta diversity based on Adonis analyses. Given the diversity between individual samples, this was not surprising.

Lower Chao 1 and Shannon index (statistically not significant) was also observed by Leuvenhaege et al. (2017) who measured differences among microbiomes from BU patients wounds compared to healthy controls and patients with other ulcers) (Van Leuvenhaege et al.,

2017). This shift of microbial community in BUD and in our data indicates that *M. ulcerans* could impact the community composition in its vicinity to determine its polymicrobial environment in its natural aquatic habitat and during infection to human host. Chao 1 accounts for species richness whereas Shannon diversity accounts for both species richness and evenness (B. R. Kim et al., 2017). The reduction in these alpha diversity indices suggests that mycolactone could either positively or negatively affect microbial flora to enrich some species whereas prohibit some other species, which would ultimately impact the overall microbial community composition and diversity, and reduce species richness and evenness in its vicinity. Higher Chao 1 in mycolactone coated slides in the non-endemic location suggests higher species richness with greater influence of rare taxa. There was no difference in beta-diversity based on locations and treatments. Although this study showed that there is no significant difference in beta diversity based on locations and treatment further study with higher sample size and including more locations will provide better insight on impact of these factors in determining microbial community composition.

Based on data from our random forest classifier, Family Comamonadaceae was the best predictor for both *M. ulcerans* endemicity and mycolactone treatment as there was higher abundance of these in endemic locations than in non-endemic locations on control slides; but also, mycolactone addition to glass slides showed increased abundance of Comamonadaceae compared to controls, no matter the location (Figure 5.9). Comamonadaceae (Phylum: BetaProteobacteria) are aerobic organotrophs or anaerobic denitrifiers and include diverse groups of bacteria capable of reducing iron, denitrification, oxidizing hydrogen and fermentation (A. Willems, De Ley, Gillis, & Kersters, 1991; Anne Willems, 2014). An increase in denitrifying and fermentative bacteria suggests that mycolactone could be positively impacting their

metabolism or other environmental conditions. More detailed investigations on the effect of mycolactone on local pH and nitrogen abundance will provide further insight on abundance of these bacteria.

Similarly, Xanthomonadaceae, Clostridiaceae, Bacillaceae and Flavobacteriaceae were in higher abundance in *M. ulcerans* endemic locations compared to non-endemic locations from control slides; and in mycolactone treated slides overall. Families Clostridiaceae and Bacillaceae within Phylum Firmicutes are generally heterotrophoic or chemoorganotrophic, and can be found in environments such as soil, feces, water, sewage and in the human gastrointestinal tract (Mandic-Mulec, Stefanic, & van Elsas, 2015; Wiegel, Tanner, & Rainey, 2006). Bacillaceae also include genera who are insect (such as *B. thuringiensis*), human and animal pathogens (such as *B. anthracis* and *B. cereus*)(Slepecky & Hemphill, 2006). (Mandic-Mulec et al., 2015; Slepecky & Hemphill, 2006). Xanthomonadaceae (Phylum Proteobacterium) include some plant pathogens (Pieretti et al., 2009), and some hydrocarbon degrading species within genera *Arenimonas*, *Luteimonas*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Xanthomonas*, and *Xylella* (Gutierrez, 2017). Flavobacteriaceae commonly inhabit marine and freshwater and soil, but some members can inhabit animal or plant hosts (McBride, 2014), causing disease in fish, birds and humans (McBride, 2014). Many genera within all of these families are chemoorganotrophs, participate in natural carbon cycling, and many genera can degrade chitin (Bowman & Nichols, 2005; Randall et al., 2020). Additionally, all of these families have genera that can utilize nitrogen for their respiration and metabolism. (Bernardet, 2015; Wiegel et al., 2006; Yousuf et al., 2017). These data suggest mycolactone may be either enhancing bacterial growth of these family members directly or may be shaping community structure in such a way as to influencing abiotic and biotic conditions promoting increased growth.

Moraxellaceae were lower in abundance in endemic locations on control slides compared to control slides from the non-endemic location but showed increased abundance on mycolactone treated slides from all locations. Moraxellaceae are found in diverse habitats of water, soil and vegetation, and have diverse functions ranging from bioremediation to being important human and animal pathogens (Teixeira & Merquior, 2014). The lower abundance of Moraxellaceae in the endemic locations could be influenced by several other biotic and abiotic factors such as niche partitioning, community interaction, organic matter content, dissolved oxygen, pH, temperature, sunlight UV exposure, aeration, etc. Moraxellaceae abundance is positively correlated with conductivity, water temperature, total phosphorus and pH whereas negatively correlated to dissolved oxygen (H. Wang et al., 2019). Further, UV exposure and aerobic environments enrich these families (Kauser, Ciesielski, & Poretsky, 2019; H. Sun et al., 2017). Since the non-endemic location is faster flowing with greater aeration, this may cause the higher abundance of Moraxellaceae in non-endemic location. But despite this, the addition of mycolactone increased Moraxellaceae abundance. Both families have diverse metabolic capabilities, and have been important for bioremediation as they can degrade hydrocarbons and other recalcitrant compounds(Teixeira & Merquior, 2014).

Sphingomonadaceae and Intrasporangiaceae were also lower in abundance in endemic locations compared to non-endemic locations from control slides, and mycolactone addition reduced their abundance further in both endemic and non-endemic sites (Figure 5.9). Interestingly, though Bradyrhizobiaceae was an important predictor Family for endemicity, as it had higher abundance in endemic locations compared to the non-endemic location, mycolactone addition reduced its abundance, no matter the location. These data suggest that mycolactone is antagonistic to these families.

Families Caulobacteraceae, Chitinophagaceae, Burkholderiaceae and Phyllobacteriaceae showed contrasting results based on treatment and endemic and non-endemic locations (Figure 5.9). Caulobacteraceae, though in higher abundance in endemic control slides compared to non-endemic controls, mycolactone treatment lowered the abundance of these in endemic locations, but increased abundance in non-endemic locations. This contrasted with families Chitinophagaceae, Burkholderiaceae and Phyllobacteriaceae that were in higher abundance in endemic control slides compared to non-endemic controls. Mycolactone treatment led to an even higher increase in these families in endemic locations, and lowered abundance in these families even further in the non-endemic location. The differences in the abundance of these families based on the location suggest greater influence of site than mycolactone in determining the interaction. This discrepancy may be caused by variation in other factors such as abundance of chitin, proximity to plants, water flow or other biotic and abiotic factors to influence interaction of mycolactone in these locations.

Members such as *Caulobacter mirabilis* and *Phenylobacterium haematophilum* within the Caulobacteraceae family can degrade surfactants such as linear alkylbenzene sulfonates whereas Phyllobacteriaceae includes member such as *Oricola Cellulosilytica* that can degrade cellulose (Cortés-Lorenzo et al., 2013; Hameed et al., 2015). Chitinophagaceae commonly inhabit soil and aquatic habitats (Dahal, Chaudhary, & Kim, 2017), are positively associated with chitin and include some chitin degrading species such as *Chitinophaga pinensis* (Randall et al., 2020). *M. ulcerans* can utilize chitin for growth, however its growth is enhanced in presence of chitin at low pH (4.5-6.5) but this effect is not seen at high pH (7.0 and 7.5) (Sanhueza et al., 2018, 2016). It is intriguing to observe the variable relationship of mycolactone with families

such as Chitinophageaceae, Phyllobacteriaceae, Caulobacteriaceae and Burkhuldariaceae. Studies have shown that various environmental factors such as substrates, temperature and pH can impact the composition of quorum sensing and quenching microbial communities (Li, Cao, & Yu, 2019). Additionally, substrate composition has shown to mediate symbiotic or antagonistic relationships between microorganisms (Deng & Wang, 2016). It is suggested that degradation of complex substrates require coordination and symbiotic relationships between microbes whereas availability of simple substrates only provokes competition between microbes to establish antagonistic relationships (Deng & Wang, 2016). The variation in microbial relationship based on environmental parameters has been shown by computational analysis for relationships between acidobacterial subgroup GP3 and GP1, where there is a positive interaction between these groups when pH and organic carbon are considered whereas weak negative interactions occur based on soil moisture content (Shang et al., 2017). Further in that analysis, the strength of interaction between these groups was higher at high pH but lower for high organic matter and moisture content (Shang et al., 2017). As plant exudates and chitin in the endemic location makes it organically rich, microbes such as Chitinophageaceae and Phyllobacteriaceae may use mycolactone as a signal to degrade those complex substrates. However, such associations for substrate utilization may not be required in non-endemic location.

Interestingly, although *M. ulcerans* is a member of phylum Actinobacteria, mycolactone showed an overall negative impact on Actinobacteria richness and evenness, such as with Nocardioidaceae, Micrococcaceae and Intrasporangiaceae. Similarly, lower relative abundance of Actinobacteria was observed for microbiomes from Buruli ulcer patients compared to healthy control, or ulcer patients with other etiology) by Leuvenhaege et al. (2017) (Van Leuvenhaege et

al., 2017). Mycolactone is a macrolide compound similar to the macrolide antibiotic erythromycin(T. P. Stinear et al., 2004; Zhanel et al., 2001). Erythromycin is produced by Actinomycetes (*Saccharopolyspora erythraea*) that inhibits several gram positive and negative aerobic bacteria as well as Actinobacteria (such as *Corynebacterium diphtheriae* and *Mycobacterium kansasii*)(Leadlay et al., 1993; Washingtion & Wilson, 1985; Zhanel et al., 2001). Both erythromycin and mycolactone are secondary metabolites and have immunomodulatory effects (Altenburg, De Graaff, Van Der Werf, & Boersma, 2010; Fred Stephen Sarfo et al., 2016). In the natural environment, these secondary metabolites (e.g. antibiotics) are produced by microorganisms to either outcompete other microbes or facilitate intracellular signaling or nutrient requisition (Salmond & Welch, 2008).

The acquisition of pMUM001 from *M. marinum,* along with loss of several genes suggests adaptation of *M. ulcerans* to a particular niche environment (Yip et al., 2007). Zingue et al (2017) showed that *M. ulcerans* utilizes several carbon sources that are related to bacteria, fungi, alga and mollusks (Zingue, Bouam, Militello, & Drancourt, 2017). Further Hammoudi *et al.* (2019) observed that mycolactone caused higher spore germination in fungi such as *Scedosporium apiospermum*, *Fusarium equiseti* and *Mucor circinelloides* and selectively attracted fungi such as *M. circinelloides* and repelled fungi such as *Fusarium equiseti* (Hammoudi, Cassagne, et al., 2019). It is suggested that the mycolactone mediated attraction of fungi could provide *M. ulcerans* with high nutrient carbon sources produced by fungi (Hammoudi, Cassagne, et al., 2019). Hence, mycolactone could serve as a *M. ulcerans* signal for resource quality and also for driving community structure by facilitating nutrient acquisition in a polymicrobial environment. It would be interesting to measure the fungal community in this

context in association with *M. ulcerans* endemicity and mycolactone treatment in natural environments.

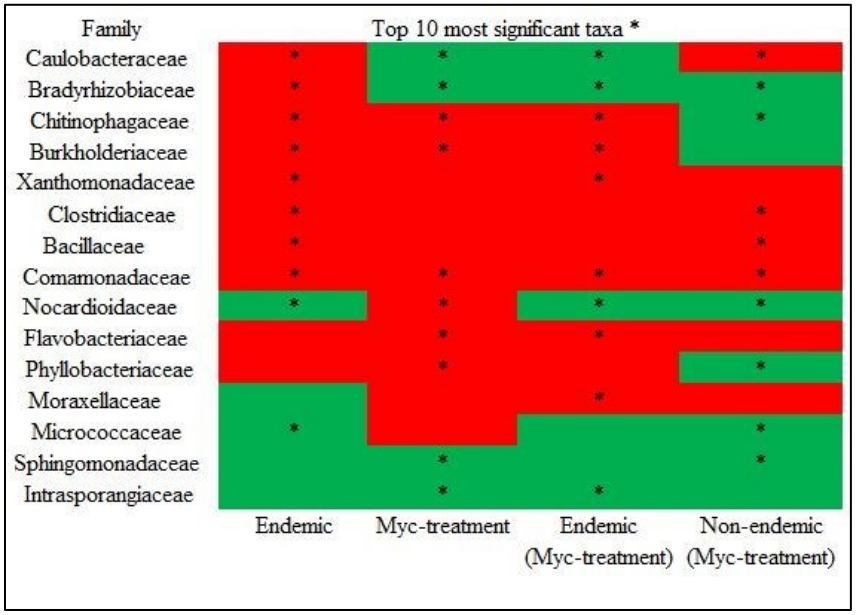

Figure 5.9 Heat map showing the top 10 most important taxa (*) and their increase (red) or decrease (green) in endemic location and in mycolactone treated slide from all aquatic location and in endemic and non-endemic locations.

(First column: Endemic location, second column: mycolactone treated slides from all aquatic locations, third column: mycolactone treated slides in endemic location, fourth column: mycolactone treated slides in non-endemic locations)

Mycolactone could also be influencing community structure by impacting cell-to-cell communication through quorum sensing or quorum quenching mechanisms. Many members within these identified families utilize quorum sensing for biofilm formation, virulence, and the production of other secondary metabolites. For instance, Comamonadaceae was found to be positively correlated with the presence of AHL (3OH-C8-HSL) in a sludge system(Panchavinin, Tobino, Hara-Yamamura, Matsuura, & Honda, 2019). They have also shown to promote biofilm

formation and degrade quorum sensing molecules (AHL) and the toxic compound toluene (C. Y. Chen, Wang, Tsai, Tsai, & Chung, 2020; Ouyang et al., 2020; Tan et al., 2014).

Comamonadaceae genera were also found to be the hub that influenced microbial interaction and diversity within the phyllosphere of *Arabidopsis thaliana* (Agler et al., 2016). They also show increased growth in the presence of an exogenous quorum quencher (Ouyang et al., 2020). Similarly, several genera within the Burkholderiaceae family such as *Burkholderia, Ralstonia* and *Pandoraea* form biofilm and use AHL as a quorum sensing molecule (Adley & Saieb, 2005; Ee, Lim, Kin, Yin, & Chan, 2014; Tabatabaei, Dastbarsar, & Moslehi, 2019; Tseng et al., 2016). *Burkholderia* is capable to form biofilm on both environment substrates and inside the human body (Rampadarath, Bandhoa, Puchooa, Jeewon, & Bal, 2017; Voronina et al., 2016). The family also includes some quorum quenchers such as *Cupriavidus* that degrades diffusible signal factor (Ye et al., 2020). *Paraburkholderia graminis,* a member in the family produces volatile sulfur compound that inhibits fungi *Rhizoctonia solani* to protect the plant against disease (Carrión et al., 2018).

Sphingomonadaceae contains several luxI/luxR pairs as well as solos of luxI and luxR in its genome(Gan et al., 2014). The members such as *Sphingomonas agrestis* and *Novosphingobium* genus produce AHLs(Gan, Buckley, Szegedi, Hudson, & Savka, 2009). The family members such as *Sphingomonas aromaticivorans* can degrade xenobiotic compounds such as naphthalene(Gan et al., 2014). Similarly, Bradyrhizobiaceae members such as *Nitrobacter winogradskyi* and *Bradyrhizobium japonicum* produce AHLs that enhance processing of nitrogen oxide gases and symbiotic relationship with other bacteria respectively(dos Santos Lima Fagotti et al., 2019; Mellbye, Giguere, Bottomley, & Sayavedra-Soto, 2016). Mycolactone disrupts lipid organization of eukaryotic membranes during its

pathology(Nitenberg et al., 2018). Further, a macrolide antibiotic Tylosin had shown to have huge and long-lasting effect on Sphingomonadaceae in a canine model (Suchodolski et al., 2009). Hence, the cell wall component of Sphingomonadaceae could make it more susceptible to macrolides such as mycolactone and may be the reason for its decreased abundance in mycolactone coated slide.

Members within the Phylum Firmicutes have been shown to respond well with the quorum sensing signals in the mammalian gut(Landman et al., 2018; J. A. Thompson, Oliveira, Djukovic, Ubeda, & Xavier, 2015). Higher abundance of Firmicutes has been related to the presence of quorum sensing molecules such as N-3-oxo-dodecanoyl homoserine lactone with two double bonds (3-oxo-C12:2)(Landman et al., 2018). Similarly, increased abundance of Firmicutes have been observed in response to the interspecies quorum sensing signaling molecule, autoinducer-2 (AI-2)(J. A. Thompson et al., 2015). Several members in the genus *Clostridium* such as *C. botulinum*, *C. perfringens*, *C. difficile* within family Clostridiaceae possess the AGR quorum sensing system that regulates sporulation and toxin production (Kumar, Mashooq, Gandham, Alavandi, & Nagaleekar, 2018). The quorum sensing system of *C. perfringens* (TI signal peptide) has shown to interfere with *hla* gene expression and production in *Staphylococcus aureus* independent of growth thereby suggesting its quorum quenching activity (Essigmann, Darkoh, McHugh, & Brown, 2017).

Family Flavobacteriaceae also includes some quorum sensing species such as *Tenacibaculum maritimum* as well the quorum quencher *Flaviramulus ichthyoenteri* (Romero, Avendaño-Herrera, Magariños, Cámara, & Otero, 2010; Y. Zhang et al., 2015). *Flavobacterium* has been shown to positively correlate with the presence of AHL (Lv et al., 2014; W. Zhang & Li, 2016). In Xanthomonadaceae, AHL and fatty acids (diffusible signal factor-DSF) act as

quorum sensing signals have been described in genera such as *Xanthomonas*, *Stenotrophomonas*, *Thermomonas* and *Lysobacter*(He et al., 2011; Huedo, Coves, Daura, Gibert, & Yero, 2018; Tan et al., 2014). The DSF mediates various functions such as motility, enzyme production, biofilm formation and virulence in *Stenotrophomona maltophilia*, an opportunistic human pathogen(Huedo et al., 2018). Similarly, in the plant pathogen *Xanthomonas oryzae* DSF enhances virulence by promoting EPS formation and xylanase activity (He, Wu, Cha, & Zhang, 2010). Additionally, DSF also function in interkingdom signaling to promote plant growth and seed germination (Huedo et al., 2018). Quorum quenching activity has been described in members within the family such as *Dyella japonica* and *Stenotrophomona maltophilia* (J. W. Chen & Chan, 2012; Huedo et al., 2018). In *S. maltophilia*, fatty acids provide quorum quenching activity against other bacteria such as *Ralstonia solanacearum* to confer competitive advantage in the environment(Huedo et al., 2018). Similarly, *Acinetobacter* spp within the family Moraxellaceae consist of quorum sensing system such as *abaI/abaR* and *anoI/anoR* that are similar to LuxI/R system in other gram negative bacteria(Mayer et al., 2018; Saipriya, Swathi, Ratnakar, & Sritharan, 2020). However, other members of *Psychrobacter* spp possess LuxR domain with absence of a LuxI-like domain despite isolation of various AHL such as 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-OH-C10 HSL in its extract, which suggests the presence of other synthase systems in the bacteria (Reen et al., 2019). Alternatively, these bacteria could use this orphan two component system to eavesdrop or hijack signals from other bacteria. In *Acinetobacter* the quorum sensing system produces several types of AHL that mediate functions such as surface-associated motility, attachment and biofilm formation (Mayer et al., 2018; Saipriya et al., 2020). The bacteria also exhibit quorum quenching activity that self-regulate its production of AHLs (Mayer et al., 2018).

Genera within these families clearly reside within a complex network within aquatic ecosystems, where chemical communication mediates nutrient cycling, competition, antagonism, and thus, community structure and functioning. In these systems, mycolactone could cause an ecological cascade event where mycolactone could be driving the extinction of one species, thereby leading to secondary extinctions of those dependent on the first for resources or communication. On the other hand, mycolactone could be enriching some species or functions within the ecosystem that facilitate nutrient acquisition and protect against adverse environmental conditions.

5.6 Conclusions

The acquisition of plasmid pMUM001 during evolution, along with its large size, suggests a specific role of mycolactone for *M. ulcerans* in the environment(Yip et al., 2007). Further, mycolactone is structurally similar to quorum sensing molecules and it has shown to continually increase after bacteria cultivation reached a plateau in an animal model(Fred Stephen Sarfo et al., 2013). Studies on evolution of *M. ulcerans* have suggested that *M. ulcerans* underwent massive genetic rearrangements, gene deletions and pseudogene formation during its evolution from its *M. marinum* progenitor, where acquisition of plasmid pMUM001 responsible for mycolactone synthesis became one of the key events during this evolution(Yip et al., 2007). Thus, acquisition of this giant plasmid for mycolactone synthesis and pseudogene formation suggests that *M. ulcerans* evolved to adapt to more restrictive niches in the environment and mycolactone could play roles in facilitating or disrupting microbial of itself or other microbes in those environments(Yip et al., 2007). As an environmental pathogen, *M. ulcerans* resides within a polymicrobial lifestyle in wounds and in aquatic habitats where its relationship with other

microbial communities could play a crucial role in providing nutrient acquisition, aiding in colonization, or dispersal in wounds and aquatic habitats.

This study was focused to understand the impact of mycolactone on determining microbial community in the environment. There was no significant difference in richness and diversity based on location and treatment. However, the phylum level classification showed positive relationships of mycolactone with Firmicutes whereas negative relationship with Actinobacteria. Similarly, among Proteobacteria, Gamma Proteobacteria had positive relationships with mycolactone whereas Alpha-Proteobacteria had negative or variable relationships and Beta-Proteobacteria had positive or variable relationships. The Bacteroidetes had both positive and variable relationships with mycolactone. These results were based when top 10 most important taxa were considered. The top 10 most important taxa based on location and treatment (overall, endemic location and non-endemic location) were determined. The most important taxa for all the categories were listed and the increase or decrease of their abundance was determined based on calculation of % OTU. There was no clear differentiation of microbial community based on the general characteristic of these families (Appendix Table 3). Most of the families included members with quorum sensing ability. However, other factors related to microbes such as their cell-wall components and individual response to quorum sensing or quenching signals can determine their affinity to mycolactone. This study was not without limitations. For instance, we had an overall small sample size that limited the power of our analyses. Also, sampling was conducted for only a few sites which included only one non-endemic location and only two sampling years within a single season. Finally, we determined how mycolactone impacted community structure. It would be interesting to further our studies in a mesocosm where viable *M. ulcerans* was added to determine active mycolactone production,

and corresponding responses. Future studies including multiple sampling sites with varying landscapes and all 4 seasons in multiple years is underway that will provide insight on influence of landscape and seasonal changes in determining polymicrobial interaction of *M. ulcerans* and mycolactone. Despite these limitations, this pilot study is a first attempt to characterize the *M. ulcerans* microbial environment and the role mycolactone may have in shaping these interactions. Altogther the integration of knowledge of signaling mechanisms with microbial members and their networks will lead to a new understanding of the fate and significance of these signals at the ecosystem level. Such an understanding could lead to new biological and chemical strategies to modulate health and productivity within aquatic sites to prevent *M. ulcerans* exposure, but also improve means to treat disease.

CHAPTER VI

RESPONSES TO CHEMICAL CROSS-TALK BETWEEN THE MYCOBACTERIUM ULCERANS TOXIN, MYCOLACTONE, AND STAPHYLOCOCCUS AUREUS

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6.1 Abstract

Buruli ulcer disease remains a 'mysterious disease' due to its unknown mode of transmission and pathogenesis. The disease is caused by the environmental pathogen, *Mycobacterium ulcerans* whose major virulence factor is mycolactone, a lipid cytotoxic molecule. Buruli ulcer has been found to be colonized by various quorum sensing bacteria such as *Staphylococcus aureus, S. epidermidis,* and *Pseudomonas aeruginosa*, but without typical pathology associated with those pathogens' colonization. *M. ulcerans* pathogenesis may not only be an individual act but may also be dependent on synergistic or antagonistic approach it exhibits within a polymicrobial network. Hence, it is important to understand the interaction of *M. ulcerans* with other bacteria encountered during skin infection. In this study, mycolactone was added to *S. aureus* and incubated for 3, 6 and 24 hours. At each time interval, *S. aureus* growth

and hemolytic activity was measured, and RNA was isolated to measure virulence gene expression through qPCR and RNASeq analyses. Results showed that mycolactone reduces *S. aureus* hemolytic activity, suppresses *hla* promoter activity, and attenuats virulence genes without inhibiting *S. aureus* growth. Analysis of RNASeq data showed mycolactone profoundly impacted *S. aureus* metabolism. The finding is significant as it aids in increasing understanding of how polymicrobial interactions within a wound might shape infectious processes.

6.2 Introduction

Buruli ulcer (BU) disease is a necrotizing skin disease whose etiological agent is *Mycobacterium ulcerans* (MU). The major virulence factor of *M. ulcerans* is mycolactone, a cytotoxic macrocyclic lipid encoded by a giant plasmid pMUM001 that was acquired during emergence from an *M. marinum* progenitor (Yip et al., 2007). Mycolactone is immunosuppressive and inhibits dendritic cell maturation and migration to the draining lymph node in mice (Coutanceau et al., 2007), inhibits other immune cells including T-cells, and suppresses cytokine and chemokine production (Sarfo et al., 2016) Buruli ulcer is endemic in West Africa, has been reported in over 33 countries worldwide and is the third most common mycobacterial infection after tuberculosis and leprosy (Babonneau et al., 2015; Borths, Poolman, Hvorup, Locher, & Rees, 2005; Yotsu et al., 2015). Fear of surgery and long distances to treatment facilities contribute to the rural poor being most negatively affected (Asiedu & Etuaful, 1998; Raghunathan et al., 2005). Though there have been recent advances in antibiotic therapy for early BU lesions (Etuaful et al., 2005; O'Brien et al., 2014), patients sometimes require extensive surgery and hospital stays, creating economic burden for families and results in patients suffering from the effects of social isolation and stigma (Amoakoh & Aikins, 2013) .Without an effective vaccine and with limited knowledge of transmission, prevention is not yet

feasible (Kotlowski et al., 2004; Merritt et al., 2010; Mosi et al., 2008; Williamson et al., 2012, 2008; Willson et al., 2013). Therefore, disease management relies on early case detection, reliable diagnosis, and early effective treatment. Alarmingly, a delay in wound healing is sometimes observed despite initiation of antibiotic therapy. Underlying mechanisms associated with this delay are not known, though hypotheses regarding the impact of mycolactone on host cells, or polymicrobial infection have been suggested (Amissah et al., 2015; Barogui et al., 2013; Fred Stephen Sarfo et al., 2016; Yeboah-Manu et al., 2013). Therefore, there is a critical need to determine *M. ulcerans* regulation and mycolactone production in response to host inoculation and infection, as well as the impact other microbial community members have on these dynamics.

Staphylococcus aureus, S. epidermidis and Pseudomonas aeruginosa have been isolated from BU wounds (Amissah et al., 2015; Barogui et al., 2013; Kpeli et al., 2016; Yeboah-Manu et al., 2013). However, disease pathology normally associated with these pathogens, such as pain, pus, or redness is strikingly absent in these ulcers, especially prior to *M. ulcerans* antibiotic treatment (Amissah et al., 2015; World Health Organization, n.d.; Yeboah-Manu et al., 2013). *Staphylococcus aureus* is a primary cause of skin and soft tissue infections, prosthetic-joint infections and infective endocarditis (Asgeirsson, Thalme, & Weiland, 2018; Challagundla et al., 2018; Olson & Horswill, 2013; Tong, Davis, Eichenberger, Holland, & Fowler, 2015), but may also be human normal flora, particularly residing within anterior nares (Archer et al., 2011). *S. aureus* expresses several factors required for colonization (adhesins) and invasion (coagulase, staphylokinase) (Kong, Neoh, & Nathan, 2016), lysis (hemolysins), immune evasion (leukotoxins), and increased pathogenicity (enterotoxins and TSST1), which are regulated by the SaeRS (*S. aureus* exoprotein expression) two component system (Baroja et al., 2016; Liu, Yeo, &

Bae, 2016; Otto, 2014); but also by the accessory gene regulator system (Agr), that regulates virulence through quorum sensing mechanisms (Gomes-Fernandes et al., 2017; B. Wang, Zhao, Novick, & Muir, 2014).

Mycolactone's mechanism on host cells has been shown to profoundly inhibit several host cytokines and chemokines that are dependent on Sec61 mediated translocation. However, cytokines such as interleukin-1B (IL-1B) which are Sec-independent and released through the caspase activated pathway, are shown to be less inhibited by mycolactone, suggesting selective inhibition of inflammatory cytokines in BU disease (Hall et al., 2014; Hall & Simmonds, 2014; Sarfo et al., 2016). Furthermore, *S. aureus* skin infection is typically characterized by abscess formation mediated by neutrophils (Miller & Cho, 2011), where the absence of pro-inflammatory cytokine IL-1B has been associated with increased lesion size in murine skin infection (Miller et al., 2008).

What is less clear is whether the absence of pus and other pathology in a co-infected BU wound is due to decreased virulence of *S. aureus* or immune suppression caused by mycolactone. Mycolactone does not appear to directly affect neutrophils in BU disease as its toxicity toward neutrophils is observed only in high doses, and absence of neutrophils in BU wounds is mainly attributed to poor neutrophil chemotaxis toward MU (Adusumilli et al., 2005; Sarfo et al., 2016).With regards to immune response related to *S. aureus,* mycolactone profoundly inhibits some cytokines such as IL-17, TNFα , IL-6 , chemokines such as IL-8 and MIP2 and adhesion molecules such as L-selectin, however, other cytokines such as IL-1α, IL-1β, chemokines such as CCCL1, CXCL5 and adhesion molecules such as P-selectin, E-selectin, intracellular adhesion molecule (ICAM1) and lymphocyte function-associated antigen 1 are less affected (Miller & Cho, 2011; Sarfo et al., 2016). Also, *S. aureus* colonizing BU wounds has been shown to contain

virulence genes such as *hla* (hemolysin), *sak* (staphylokinase), and *luk* (several leukocidins including *lukD*, *lukE*, *lukF*, *lukS*, and PVL) with a potential to cause serious and invasive infections. However, absence of any pus or invasive infection in BU patients colonized with *S. aureus* suggests attenuation of *S. aureus* virulence in BU disease and a potential role of mycolactone in driving this attenuation (Amissah et al., 2017, 2015).

The macrocyclic structure of mycolactone is similar to quorum sensing (QS) compounds in other bacteria. Studies have shown that some QS molecules such as 3-oxo-C_{12}-Homoserine lactone produced by *P. aeruginosa* can inhibit QS systems of other bacteria, such as inhibition of *S. aureus sarA* and *agr* expression (Qazi et al., 2006). The inability of isolated pathogens such as *S. aureus* and *P. aeruginosa* to cause disease in BU wounds is intriguing and suggest mechanisms of *M. ulcerans* to attenuate the virulence of those pathogens during infection. Therefore, the purpose of this study was to understand the effect of mycolactone on the expression of *S. aureus* virulence factors and hemolysis.

6.3 Materials and Methods

6.3.1 Overall approach

Overnight grown *S. aureus* 502a (Parker et al., 2014) was transferred to Luria Bertani (LB) broth such that the final OD600 was 0.25. Synthetic mycolactone A/B (donated from Yoshito Kishi's laboratory, Department of Chemistry and Chemical Biology, Harvard University) was diluted to concentrations of 50ng, 100ng and 300ng/mL in ethanol, dried down, then added in a 5µL volume (in ethanol, EtOH) to respective flasks containing *S. aureus*, then incubated at 37° C. Ethanol (5µL) was also added to separate flasks containing *S. aureus* as a solvent control. At 3H, 6H, and 24H of incubation, samples were taken for measurement of growth (OD600), hemolytic activity, and relative quantitation of *agrA, saeR* and *hla* gene

expression (Figure 6.1). Experiments were performed at least three times and in sample triplicate.

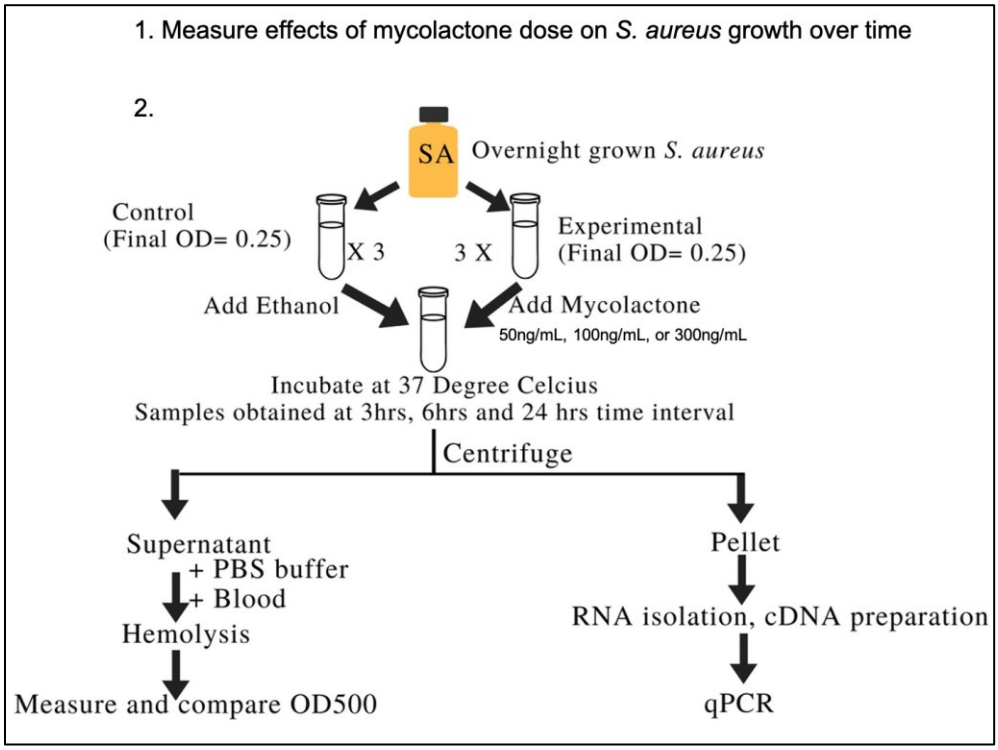

Figure 6.1 Overall approach to determine the effect of mycolactone on S. aureus growth, hemolytic activity and virulence gene expression.

6.3.2 Measurement of *S. aureus* hemolytic activity

Hemolytic activity of *S. aureus* incubated with mycolactone was measured and compared with *S. aureus* containing ethanol using a modified pneumolysin hemolysis assay (Bryant et al., 2016). Briefly, dilution buffer containing phosphate buffered saline (PBS) and bovine serum albumin (BSA) was prepared and added to a 96 well V-bottom plate. *S. aureus* supernatant was filtered through a 0.2-micron filter and 100µl was added to respective wells and serially diluted in a 1:2 dilution. Fifty microliters of PBS washed rabbit blood was added to each well and

incubated at 37° C for 1H. Distilled water was used as a positive control for hemolytic activity and buffer was used as a negative control. After incubation, the plate was centrifuged at 1000 x g and supernatant was carefully transferred to a 96 well flat bottom plate where OD540nm was measured to determine the hemolytic activity of *S. aureus* incubated alone or with mycolactone or EtOH, relative to the distilled water + red blood cell lysis positive control and to *S. aureus* + ethanol control.

6.3.3 RNA isolation

RNA was isolated using the Trizol method according to manufacturer's instructions. Briefly, bacterial cell suspensions were centrifuged and Trizol reagent was added to the pellet, mixed thoroughly, and homogenized with 0.2mm beads. After incubation, 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. The RNA was cleaned using the Qiagen RNeasy PowerClean Pro Cleanup Kit. RNA quality was analyzed by agarose gel electrophoresis and RNA concentrations were determined using Qubit 2.0. RNA was treated with Turbo DNAse (Invitrogen) according to the manufacturer's instructions, to remove trace DNA as necessary. All samples were stored at -80°C until further processing for cDNA preparation and RT-qPCR, or for library preparation (described below).

6.3.4 *S. aureus* cDNA preparation

S. aureus cDNA was prepared using the Verso enzyme kit (Thermo Scientific) according to the manufacturer's instructions. The reaction mixture for cDNA preparation included 4µl

synthesis buffer, 2µl dNTP mix, 1µl random hexamer, 1µl Verso enzyme and 1µl RT enhancer and the template. The reaction mixture was heated at 42°C for 1H to obtain cDNA.

6.3.5 Quantitative real time PCR (RT-qPCR)

RT-qPCR was performed targeting *agrA, saeR* and *hla* genes. The Shikimate dehydrogenase (*aroE*) gene was used as a housekeeping gene, and appropriate positive and negative controls were included in each run. The master mix contained 1.0µl of each forward and reverse primer for *aroE* gene and target gene, 2.0µl of target probe and *aroE* probe, 12.5µl of master mix, 0.5µl water and 3.0µl template cDNA per well of PCR plate. PCR was conducted using a BioRad CFX96 with the following parameters: 95°C for 10 minutes, and 39 cycles of 95°C for 15 seconds, and 57°C for 30 seconds for the *hla* gene and 39 cycles of 95°C for 15 seconds or 59°C for 30 seconds for *agr*A and *sae*R genes, respectively. The sequences of forward and reverse primers used for each gene are listed in Table 6.1.

Table 6.1 Primer Sequences of Genes Amplified in RT-qPCR

Primers	Sequence
Forward *aroE*	5'ATGGCTTTAATATCACAATTCC3'
Reverse *aroE*	5'CTATCCACTTGCCATCTTTTAT3'
Forward *agrA*	5'TCACAGACTCATTGCCCATT3'
Reverse *agrA*	5'CCGATGCATAGCAGTGTTCT3'
Forward *saeR*	5'GCTAAATACCACATAACTCAAATTCC3'
Reverse *saeR*	5'TTGAACAACTGTCGTTTGATGA3'
Forward *hla*	5'GTGTATGACCAATCGAAACATTTGCA3'
Reverse *hla*	5'GGTAATGTTACTGGTGATGATACAGGAA3'

6.3.6 Library preparation, RNA seq and analysis

RNA libraries were created from combined triplicate RNA samples *of S aureus* controls and *S. aureus* with mycolactone supplementation (300 ng/mL concentration) at the above timepoints. 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 (Krueger, 2015) (Krueger, 2015), 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. Resulting high-quality reads were aligned to the *S. aureus* NZ_CP007454 reference genome (downloaded from the NCBI database). 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. Differential expression was measured between *S. aureus*-mycolactone treatment against *S. aureus*-EtOH control for the 3H, 6H, and 24H timepoints in the CLC Workbench. The program uses multi-factorial statistics based on a negative binomial generalized linear model. Treatment reads with an absolute fold change of 1.5 or higher, and FDR adjusted p-value less than or equal to 0.05 were considered significant. Transcripts were further annotated into pathways by linking protein ID with potential conserved domains and protein classifications archived within the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), and by using the UniProt, KEGG and STRING databases (Consortium, 2018; Kanehisa, Furumichi, Tanabe, Sato, &

Morishima, 2017; Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016; Szklarczyk et al., 2015).

6.3.7 Impact of mycolactone on *hla* promoter activity

To monitor *hla* promoter activity, the *hla* promoter was amplified and transcriptionally fused with the LuxABCDE operon in a pMK4 vector (Francis et al., 2000). The luminescence reporter plasmid was electroporated into *S. aureus* LAC (Kennedy et al., 2008, 2010) strain harboring the luminescence reporter plasmid and was cultured in brain heart infusion broth supplemented with 50-500 ng of mycolactone or vehicle control (DMSO) at 37^{o}C for 24H. The luminescence signal was monitored by Cytation 5 (BIoTek).

6.3.8 Statistical analysis

Significant difference in growth, percent reduction in hemolysis, and *hla* promoter activity (luminescence) of *S. aureus* containing mycolactone compared to *S. aureus*-EtOH control was determined using one-way analysis of variance (ANOVA). The RT-qPCR data was analyzed using Python code implementing the ΔΔCT method to determine fold change relative to housekeeping control and significant difference (P-value<0.05) (Livak & Schmittgen, 2001). Resulting regulation was determined relative to control samples, which was considered baseline. A fold change greater than 1 was considered as upregulated. For fold change between 0 to 1, the negative of the reciprocal of fold change was calculated to determine downregulation.

6.4 Results

6.4.1 *S. aureus* growth is not inhibited by mycolactone

S. aureus was incubated with mycolactone (concentration 0 ng – 10 μg) over time to determine whether there was any effect by mycolactone on *S. aureus* growth. Based on optical

density (OD600nm), low to moderate mycolactone concentrations (1ng-1µg) had no effect on *S. aureus* growth for up to 16H, though 10 µg mycolactone had some effect on S. *aureus* growth after 7H (Figure 6.2A). There was no statistical difference in growth between *S. aureus* containing 0, 50, 100 and 300 ng/mL mycolactone and control at 3H, 6H and 24H timepoints (Figure 6.2B).

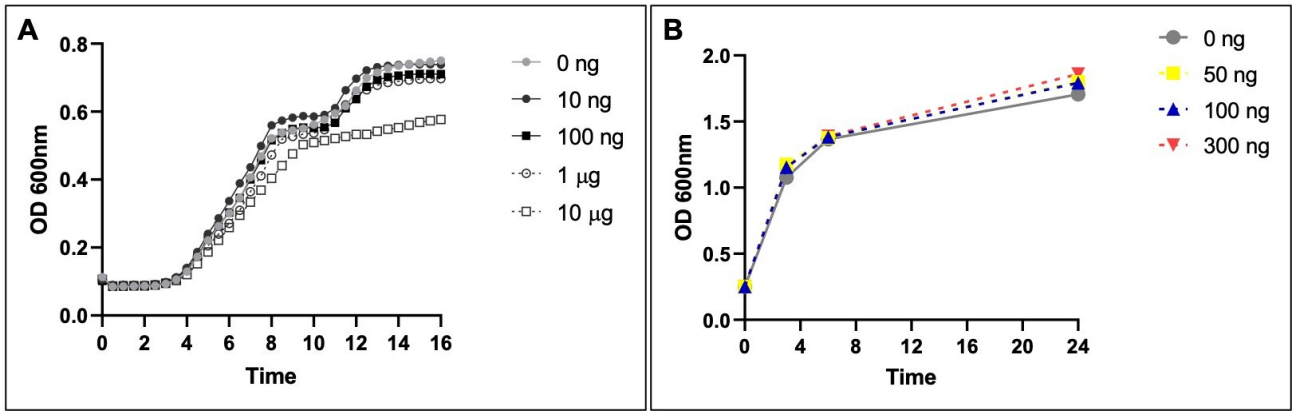

Figure 6.2 Effect of mycolactone on *S. aureus* growth

(A) Dose effect of mycolactone on *S. aureus* growth measured by OD600 for up to 16 hours. (B) Comparison of OD600 of *S. aureus* incubated alone, or with mycolactone or EtOH at 3H, 6H, and 24H.

6.4.2 Mycolactone reduces *S. aureus* hemolytic activity

S. aureus hemolytic activity was significantly decreased for *S. aureus* incubated with mycolactone (concentration 50, 100 and 300 ng) compared to *S. aureus* incubated with EtOH at 3H. While hemolysis was decreased for *S. aureus* incubated with all concentrations at 6H compared to controls, this decrease was not statistically significant. At 24H, hemolysis was only significantly reduced for *S. aureus* incubated with 300ng mycolactone (Figure 6.3).

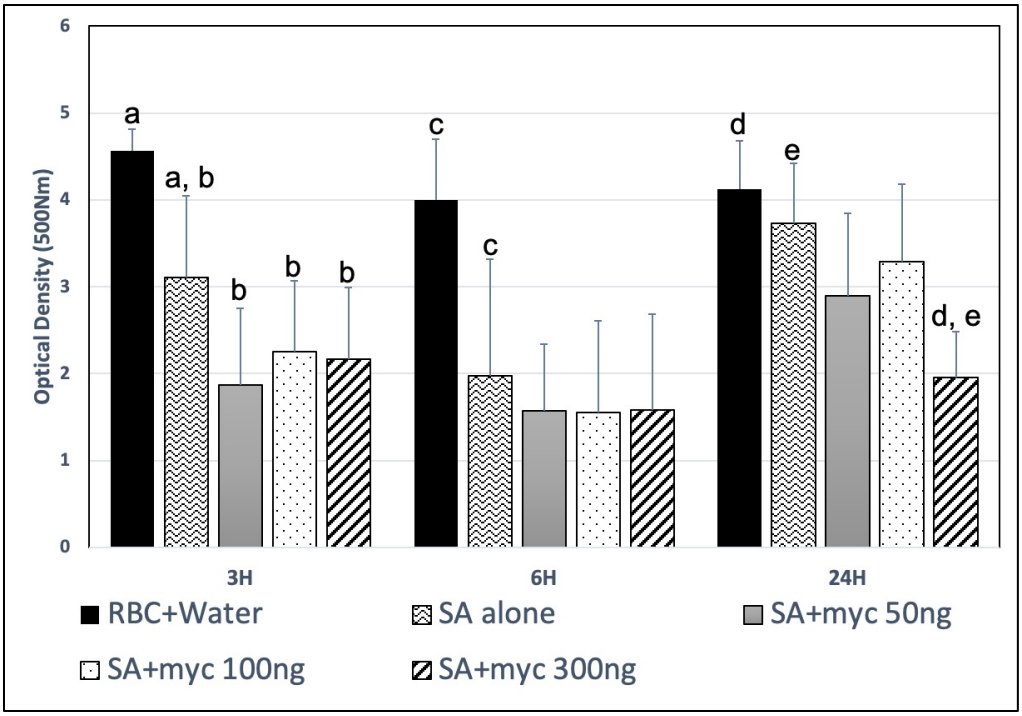

Figure 6.3 Mycolactone co-Incubation reduced *S. aureus* hemolysis over time.

Graph showing OD500 of red blood cells incubated with water (positive control), *S. aureus* alone, *S. aureus* plus 50ng mycolactone, *S. aureus* plus 100ng mycolactone, or *S. aureus* plus 300ng mycolactone. Significance was determined as <0.05. a,b, c, and d,e denotes significantly different treatments at 3H, 6H, or 24H, respectively.

6.4.3 Mycolactone leads to modulation of *S. aureus* global regulator gene expression

The effect of mycolactone on *S. aureus agrA*, *saeR* and *hla* virulence genes was determined by RT-qPCR. The results showed that *agrA* was not significantly modulated at 3H (Figure 6.4A) but was significantly downregulated for *S. aureus* incubated with mycolactone (300ng at 6H ($p=0.043$, Figure 6.4B). However, *agrA* returned to control levels at 24H (Figure 6.4C). RT-qPCR of *sae*R gene activity showed no statistical difference from control at 3H (Figure 6.4D), but, *sae*R was significantly downregulated at 6H for the 300 ng mycolactone treatment ($p= 0.029$, Figure 6.4E) and at 24H ($p=0.04$ for 100ng and $p=.006$ for 300 ng, Figure 6.4F). Similarly, the *hla* gene was not significantly different from control at 3H (Figure 6.4G),

but was significantly downregulated for *S. aureus* incubated with mycolactone (300ng) at 6 hours (p=0.04, Figure 6.4H) and at 24 hours (100 ng, p=0.03 and 300 ng, p=0.05, Figure 6.4I) compared to control.

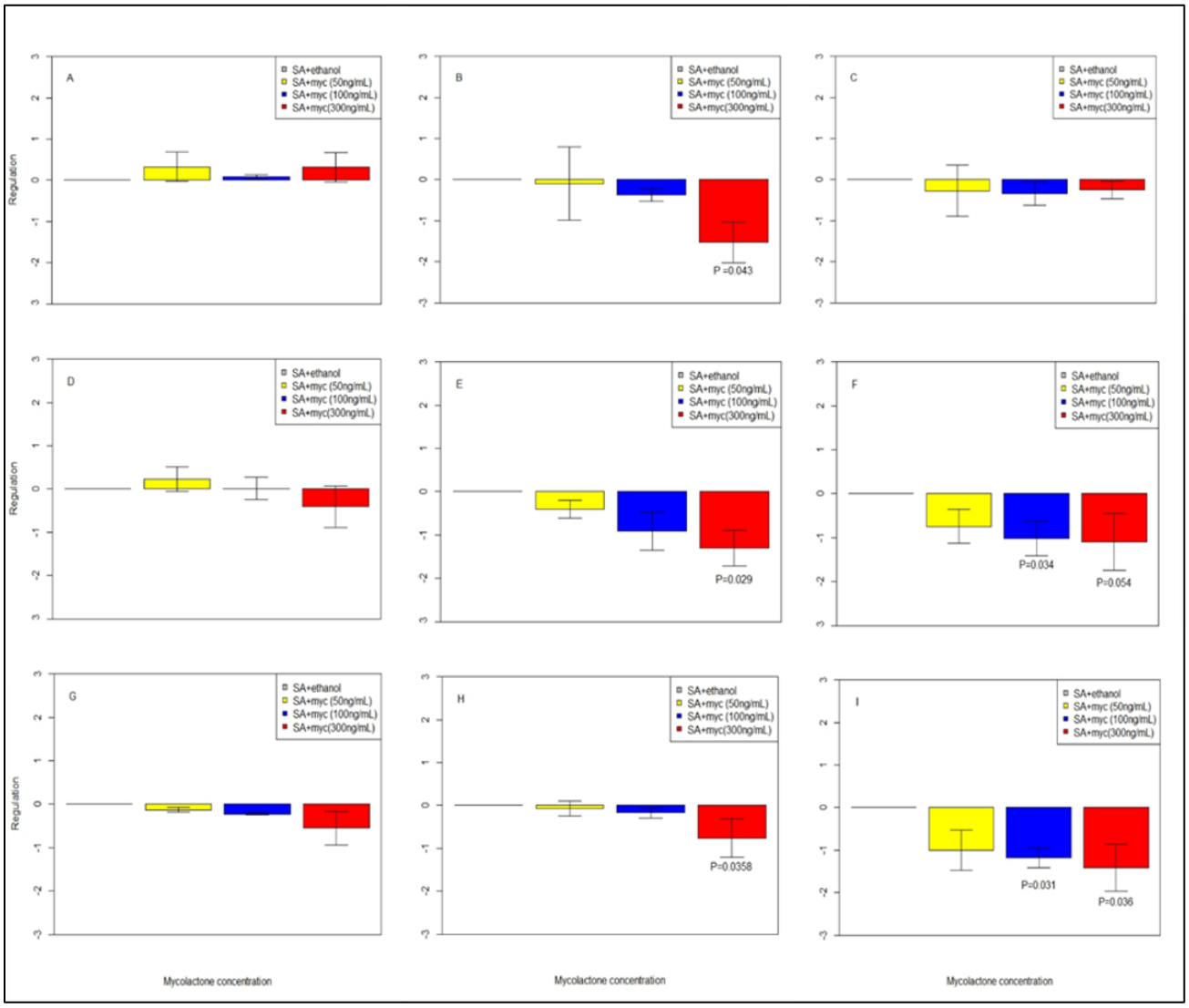

Figure 6.4 Gene expression of S. *aureus agr*A, *saeR*, and *hla* when exposed to 50, 100, or 300 ng mycolactone concentrations over time.

(A-C) *agr*A regulation at 3H, 6H, and 24H, respectively. (D-F) *sae*R regulation at 3H, 6H, and 24H, respectively. (G-I) *hla* expression at 3H, 6H, and 24H respectively. Regulation was measured for all genes against the *aroE* housekeeping gene.

6.4.4 Mycolactone suppresses *hla* promoter activity

We tested a range of mycolactone (50 to 500 ng) supplementation with *S. aureus* on promoter activity of the *hla* gene. Mycolactone at 100 ng was the minimal concentration to suppress *hla* gene expression across our tested timepoints. Significant suppression of *hla* promoter activity occurred between 3.5-6.5H, 9H-12.5H, and 14-24H for the *S. aureus*+100ng mycolactone treatment (Appendix Figure 1). At 4 hours, the mean percent difference in luminescence between the vehicle control and mycolactone treatment was -57% (p=.004, Figure 6.5). Luminescence in the mycolactone treatments continued to be lower than vehicle control at 8H (-24%, p=0.24), 12H (-91%, p=0.01), 16H (-44%, p=0.007), 20H (-135%, p=0.0008), and 24H (-211%, p=0.0002). *S. aureus*+ 200ng mycolactone significantly suppressed *hla* promoter activity between 2H-13.5H, then between 15.5-24H (Appendix Figure 1). At 4H, the mean percent difference in luminescence between the mycolactone treatment and vehicle control was -25% (p=0.370). Luminescence in the mycolactone treatments remained less than that from the vehicle control for 8H (-37%, p=0.05), 12H (-112%, p=0.008), 16H (-44%, p=0.024), 20H (-78%, p=0.014), and 24H (-137%, p=0.001, Figure 6.5). Mycolactone with 300ng concentration suppressed *hla* promoter activity at 1.5H, between 6.5-12.5H, at 15H, and between 16H-24H (Appendix Figure 1). The mean percent difference in luminescence between mycolactone treatment and vehicle control was 9% (p=0.720) at 4H, -94% (p=0.03) at 8H, -77% (p=0.0008) at 12H, -44% (p=0.041) at 16H, -149% (p=0.004) at 20H, and -162% (p=0.001) at 24H. Mycolactone at 400ng significantly suppressed *hla* promoter activity at 2H, between 7.5H-8H, 9.5H-12H, and 16.5H-24H (Appendix Figure 1). Mean percent difference in luminescence of mycolactone treatments compared to control was 17% (p=0.37) at 4H, -311% (p=0.02) at 8H, -67% (p=0.036) at 12H, -53% (p=0.101) at 16H, 152% (p=0.007) at 20H, and -106% (p=0.008) at

24H. Finally, 500ng mycolactone significantly suppressed *hla* promoter activity between 7.5H-10.5H then 17H-24H (Appendix Figure 1). Luminescence was 15% greater in mycolactone treatments than controls at 4H (0.54). But luminescence decreased in mycolactone treatments compared to controls at 8H (-131%, p<0.001), 12H (-4%, p=0.46), 16H (-8%, p=0.42), 20H (-144%, p=0.01), and 24H (-145%, p=0.02). Significance percent differences at specific timepoints is indicated in Figure 6.5 with a star.

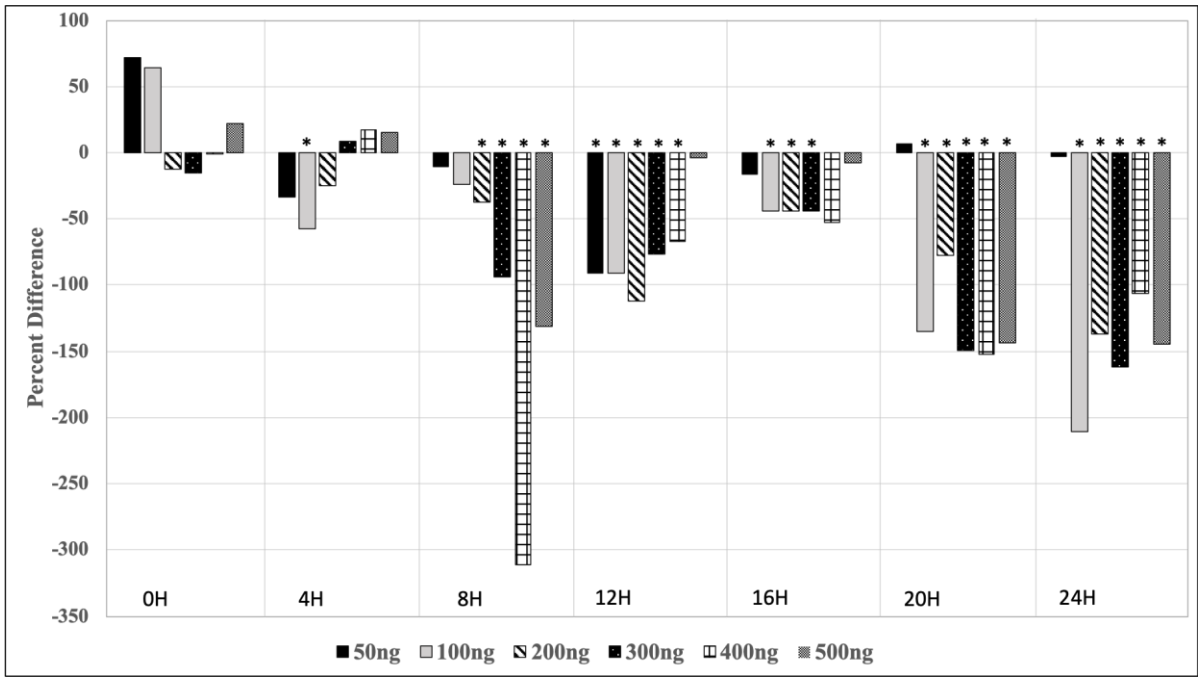

Figure 6.5 Percent Difference in *hla* Promoter Activity (Luminescence) of mycolactone Treatments from Vehicle Control.

6.4.5 Mycolactone modulates *S. aureus* global gene expression

Sequencing and trimming yielded an average fragment length of 143, and between 53 and 101 million reads per sample. To measure the effects of mycolactone on *S. aureus* global gene expression, transcriptome studies were performed using RNASeq analysis. Altogether, 122

genes were significantly differentially regulated in the *S. aureus*-mycolactone treatments in comparison to the *S. aureus*-EtOH controls (Appendix Figure A.2). Complete gene lists of significantly up-or down-regulated genes are shown in Supplemental Table 1. Sixty-nine genes were significantly up regulated, and fifty-three genes were significantly downregulated in response to mycolactone in comparison to control.

6.4.5.1 Three-hour timepoint

Forty-five differentially regulated genes were identified at the 3H timepoint. These included twenty-one downregulated and twenty-four upregulated genes in the *S. aureus*-mycolactone treatment compared to control. Fourteen genes associated with metabolism were differentially regulated (Figure 6.6). For instance, a thioredoxin reductase (*trxB*), involved in amino acid metabolism was downregulated, while two genes for arginine biosynthesis (*argG* and *argH*) were upregulated. One gene *fakb2*, was upregulated, and involved in lipid metabolism.

Three genes involved in metabolism of cofactors/vitamins were upregulated including genes involved in folate (*dfrA*), thiamine (*thiD*), and vitamin B6 (*pdxS*) metabolism. Carbohydrate metabolism was modulated where two genes, *adhE* and *adhP*, encoding for alcohol dehydrogenase were downregulated as was one gene for L-lactate dehydrogenase (LDH). The *ulaB* gene, encoding the PTS lactose transporter subunit IIB, was upregulated. Three genes for energy metabolism were downregulated including *qoxA*, *qoxB*, and *qoxC,* genes for probable quinol oxidase subunits. However, nitrate reductase beta chain *narH*, involved in nitrogen metabolism, was upregulated (Figure 6.6).

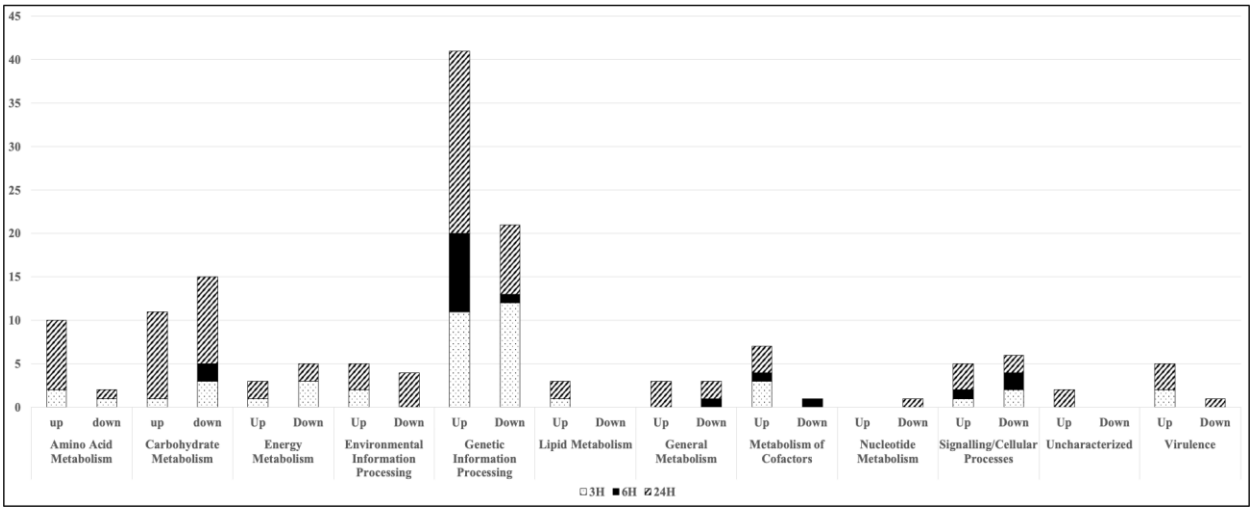

Figure 6.6 Number of genes significantly modulated in mycolactone treatments compared to controls according to functional category and timepoint.

Twelve genes involved in genetic information processing were downregulated at the 3H timepoint (Figure 6.6). These included 7 genes involved in mechanistic components of translation, one for transcription, and three transcriptional regulators including the transcriptional activator *sarR*, a negative regulator of *sarA* transcription and positive regulator of expression of primary transcripts RNAII and RNAIII generated by the Agr locus. Genes encoding a universal stress protein (*uspA*) and a cold shock protein (*cspC*), along with the preprotein translocase subunit *secY* were also downregulated. Among those upregulated within the genetic information processing category included the transcriptional activator, *rinB*, *gtfB,* encoding a stabilizing protein that is part of the accessory SecA2/SecY2 system, the repair gene *recR*, and 8 genes involved in mechanistic components of translation.

Genes encoding a probable potassium-transporting ATPase B chain gene (*kdpB*) and a glutamine ABC transporter ATP-binding protein (*glnQ*), both involved in environmental information processing were upregulated. Also, the signaling and cellular processing genes

encoding L-lactate permease (*lldP*) and xanthine permease (*pbuX*) were downregulated while *sspA*, encoding a V8-like Glu-specific serine protease was upregulated. Finally, surface protein A (Borths et al.) and clumping factor A (*clfA*), both involved in virulence, were upregulated at the 3-hour timepoint.

6.4.5.2 Six-hour timepoint

Eighteen genes were significantly modulated at the 6H timepoint, including six downregulated and twelve upregulated. These included downregulation of *adhE*, and also of *wecB*, a UDP-N-acetylglucosamine 2-epimerase involved in capsule synthesis. The gene encoding L-lactate permease (*lldP*) also remained downregulated. Additionally, *floA*, encoding scaffold protein flotillin and a gene encoding an acyltransferase were also downregulated. Genes *recR* and *rinB* remained significantly upregulated, as did *sspA*, and genes for thiamine and vitamin B6 metabolism. Seven genes involved in mechanistic components of translation were also upregulated while one was downregulated (Figure 6.6).

6.4.5.3 Twenty-four-hour timepoint

Ninety-one significantly and differentially regulated genes were identified at the 24H timepoint. These included thirty-one downregulated and sixty upregulated genes in the *S. aureus*-mycolactone treatment compared to control. Forty-four genes associated with metabolism were differentially regulated (Figure 6.6). Eight genes involved in amino acid metabolism were upregulated including members of the histidine utilization pathway (*hutU*, *hutG*, *hutI*), those for arginine and proline metabolism (*gdhA*, *rocD*, *pruA*, *putA*), and for aspartate family biogenesis (*lysC*). The only downregulated gene in this category was *trxB* (thioredoxin reductase). Both *lip1* and *lip2,* encoding lipases were upregulated. And, genes

involved in general (*pnbA*, and a gene encoding diapolycopene oxygenase, and *pfla*, pyruvate formate lyase activating enzyme), menaquinone (*menC*) thiamine (*thiD)*, and vitamin B6 (*pdxS*) metabolism were also upregulated, while a gene for nitric oxide deoxygenase (*hmp*) and a gene for ribonucleoside-diphosphate reductase subunit beta (*nrdF*) was downregulated. Two genes for energy metabolism were upregulated including a gene encoding ATP F0F1 synthase subunit beta, and *yrp,* a nitronate monooxygenase. Downregulated genes involved in energy metabolism included *narZ* and *arcC*, both involved in nitrogen metabolism. Finally, twenty genes involved in carbohydrate metabolism were modulated. These included 10 genes downregulated involved in glycolysis or gluconeogenesis, the pentose phosphate pathway and anaerobic respiration and ten upregulated genes involved in gluconeogenesis or the TCA cycle.

Twenty-nine genes were differentially regulated that are known to be part of genetic information processing. These included downregulation of both copies of the cold shock protein (c*spC*), *hup,* involved in DNA replication and repair, *csbD*, involved in stress response, and 3 genes involved in transcription and translation mechanics. The remaining genes were upregulated. One is known to be involved in DNA repair (r*ecR*), seven are chaperones (*dnaJ, dnaK, groEL, groES, grpE, clpX and clpB*), three are transcriptional regulators (*rinB*, *ctsR* and *hrcA*), one encodes a general stress protein (*yzzA*), and two are involved in ribosome biogenesis.

Three virulence genes were upregulated, including clumping factor a (*clfA*), serine-aspartate repeat-containing protein C (*sdrC*), and surface protein F (*sasF*) while surface protein G (*sasG*) was downregulated. Five genes involved in signaling and cellular processes were modulated, including downregulation of *ptr2*, encoding a peptide ABC transporter permease and a LysM peptidoglycan-binding domain-containing protein. However*, sspA*, UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase GtfA subunit (*gtfA*)t, and a

phosphate starvation-inducible protein PhoH (*phoH*) were upregulated. Seven genes were modulated involved with environmental information processing including upregulation of *mcsA* and *mcsB*, both part of the clpC operon and required for stress tolerance as well as upregulation of the alkaline shock response membrane anchor protein AmaP (*amaP*) (Wozniak, Tiwari, Soufan, & Jayaswal, 2012). Genes downregulated in this category included those involved in zinc transport (*znaB* and *znaC*), nitrate transport (*narT)*, and an autolysin (*aaa*). Finally, two uncharacterized genes were differentially upregulated at the 24H timepoint (Figure 6.6).

6.5 Discussion

S. aureus possesses an arsenal of cell-associated and extracellular virulence factors, exotoxins, enterotoxins, and superantigens (Kane, Carothers, & Lee, 2018). Many of these are regulated by SaeRS and Agr and including α-toxin (encoded by *hla*), contribute to bacterial invasion, and resistance and evasion to host defense mechanisms (Kane et al., 2018). However, despite this array of virulence factors, coinfection of *S. aureus* in a BU wound does not elicit a pathological response typical to *S. aureus* wound infections. Studies have shown that QS and other secondary metabolite molecules of one bacterium can serve positive and negative regulatory roles in cell-to-cell communication in unrelated organisms (De Kievit & Iglewski, 2000). Additionally, macrolide antibiotics have been shown to act as QS, virulence gene and biofilm antagonists at subinhibitory concentrations (Gillis & Iglewski, 2004; Gui et al., 2014; Nalca et al., 2006; Sofer, Gilboa-Garber, Belz, & Garber, 1999; Tateda et al., 2001). Mycolactone is a polyketide derived macrolide produced by *M. ulcerans* that is also structurally similar to QS compounds in other bacteria, leading us to determine whether mycolactone attenuates virulence of other pathogenic bacteria during skin co-infection. In doing so, our objectives were to determine effects of mycolactone on *S. aureus* growth over time, measure *S.*

aureus hemolysis activity following mycolactone supplementation, measure modulation of targeted *S. aureus* virulence and global regulators, and to compare global gene expression using RNASeq.

Our RT-qPCR data show that mycolactone downregulates *S. aureus* global response regulators *saeR* and *agr*A, as well as *hla* in a dose dependent manner. We have also shown that mycolactone attenuates hemolytic activity and suppresses *hla* promoter activity. Further, mycolactone elicits these responses without inhibiting *S. aureus* growth, except at very high, nonclinically-relevant concentrations (Sarfo et al., 2014; Sarfo et al., 2016). This mycolactone targeting of genes and cellular processes responsible for pathogenesis and virulence rather than those necessary for growth are expected to impose a less restrictive selective pressure on *S. aureus*. It will thus be interesting to dig deeper into whether *M. ulcerans* uses mycolactone for targeting of *S. aureus* social activities within a wound, and how that might impact microbial community ecology and resulting pathologies within that context.

The Agr system is responsible for expression of over 70 *S. aureus* genes, including many secreted virulence factors such as leukocidins, enterotoxins, lipases, and exoproteases and is also responsible for detachment of biofilms (Boles & Horswill, 2008; Miller & Bassler, 2002; Roux, Todd, Velázquez, Cech, & Sonenshein, 2014; Thompson & Brown, 2017; Yarwood & Schlievert, 2003). SaeR is the response regulator that is part of the major Sae global regulator system of many *S. aureus* virulence genes (Novick, 2003). The Sae system regulates the expression of α-toxin by binding to the consensus SaeR-binding site upstream of the *hla* promoter, though *hla*, as well as other genes regulated by Sae can also be regulated by multiple regulators containing the SaeR binging sequence, such as the P1 promoter of both *SaeRS* and *hla* to promote autoinduction (via P1 promoter) and virulence (Liu et al., 2016; Morfeldt, Taylor, Von Gabain, & Arvidson, 1995;

Nygaard et al., 2010; Xiong, Willard, Yeaman, Cheung, & Bayer, 2006). Despite our findings, mechanisms by which mycolactone suppress transcription of the *hla* gene remain elusive. One possibility is that mycolactone might compete with Agr dependent autoinducing peptide which upregulates transcription of *hla* at the stationary phase of growth (Novick & Geisinger, 2008). The other possibility is that mycolactone might bind to the sensor domain of two component regulatory systems such as SaeRS and AgrAC and block the downstream signal cascade. Further study is necessary to determine these possibilities.

But mycolactone is also known to diffuse rapidly and passively through the plasma membrane within target eukaryotic cells. Besides cytopathic effects, mycolactone exerts immunosuppressive effects by blocking co-translational translocation of inflammatory mediators through direct interaction with the α-subunit of the Sec61secretory system, resulting in protein translation in the cytosol where they are degraded by the proteasome (B. S. Hall et al., 2014; B. Hall & Simmonds, 2014). This activity also results in a lack of inflammatory infiltrates in ulcerative lesions.

Similar secretion systems such as SecYEG are present in prokaryotes, which are responsible for secretion of several proteins (Mandon, Trueman, & Gilmore, 2009). In *S. aureus,* these secretion systems are involved in secretion of several toxins and virulence factors (Sibbald et al., 2010). So, the question arises of whether the effect of mycolactone on protein secretion is limited to eukaryotes or does it also extend to prokaryotes? Our RNASeq data showed the gene encoding preprotein translocase subunit SecY was significantly downregulated. This protein is part of the protein translocation channel required for secretion of some exported proteins beyond the cytoplasm to the cell surface or to secrete proteins out of the bacterium (M. Braunstein, Brown, Kurtz, & Jacobs, 2001; Miriam Braunstein, Espinosa, Chan, Belisle, & Jacobs, 2003; Lenz,

Mohammadi, Geissler, & Portnoy, 2003). The gene *floA*, encoding flotillin that assists in assembly of membrane components and in the type VII secretion system was also downregulated (Mielich-Süss et al., 2017). These data suggest that mycolactone might also be blocking protein secretion pathways in *S. aureus.* Though this requires further investigation.

Despite this, the up regulation of genes encoding surface proteins ClfA, SdrC, SasF, and Spa, which promote the adhesion of *S. aureus* suggests that mycolactone supplementation induces mechanisms for biofilm formation. This mechanism for protection during antibiotic stress has been shown in other *S. aureus* studies (Liu et al., 2018; Paharik & Horswill, 2016). However, we did not measure biofilm formation or biomass in this experiment, and this would be a logical next step. But we have data showing that *S. epidermidis* incubated with mycolactone have increased biofilm formation (data not shown). Furthermore, work should be conducted to determine whether significantly induced gene expression corresponds to protein production.

Our RNASeq data were also consistent with oxygen limitation when *S. aureus* was supplemented with mycolactone. This was reflective in a significant reduction in expression of three quinol oxidase subunit genes and an increase in *pflA* a pyruvate formate lyase activating gene, and a nitrate reductase gene (*narH)*, But, other genes involved in fermentation and nitrogen metabolism, including genes such as *ldh*, *lldp*, *adhP*, *adhE*, *narT* and *narZ* were downregulated. Also, almost every facet of metabolism was altered by mycolactone. These included amino acid, carbohydrate, nucleotide, energy, and lipid metabolism (Figure 6.6). Genes involved in ribosome biogenesis and translation were also differentially affected.

Carbohydrate and energy metabolism particularly in gluconeogenesis and the tricarboxylic acid (TCA) cycle were expressed at a greater level. Gluconeogenesis uses phosphoenolpyruvate (PEP) as a starting substrate, which can be generated from tricarboxylic acid (TCA) cycle

intermediates. Additionally, genes for arginine, proline and aspartate family biosynthesis were upregulated, as were genes for histidine catabolism. These results suggest a metabolic flux where *S. aureus* was differentially regulating the flow of nitrogen and carbon through the cell in response to mycolactone. Also, the arginine pathway has been shown to be important for *S. aureus* persister cell formation for antibiotic and stress tolerance as well as successful survival on host skin during infection (Thurlow et al., 2013; Yee, Cui, Shi, Feng, & Zhang, 2015).

The amphipathic nature of mycolactone suggested that it may also exert its activity against *S. aureus* by perturbing membrane function and has other toxic effects leading to induction of genes involved in stress response. Indeed, a recent study outlined mycolactone's preference for membrane relative to aqueous environments (López, Unkefer, Swanson, Swanson, & Gnanakaran, 2018). Furthermore, our RNASeq data point to signs of significant response to mycolactone supplementation. Lipases *lip1* and *lip2* were upregulated. Interestingly, *fakb2* that preferentially binds unsaturated fatty acids for their uptake was also upregulated (Krute, Ridder, Seawell, & Bose, 2019). Stress genes *McsAB* and the alkaline stress response gene *amaP* were upregulated. Also, known cell wall stress stimulon members including *clpC*, *clpB*, and *clpX*, in addition to genes encoding the major heat shock proteins *GroE*L, *GroES*, *DnaK*, and *DnaJ* had significantly increased expression. Treatment of *S. aureus* with cell wall-active antibiotics is believed to result in the accumulation of damaged, misfolded, and aggregated proteins, and is also likely to be the same in the presence of mycolactone (V. K. Singh, Jayaswal, & Wilkinson, 2001; Straus & Hancock, 2006). But, *hrcA* and *ctsR* encoding proteins that negatively regulates gene expression of these loci were also upregulated (Chastanet, Fert, & Msadek, 2003). This paradoxical upregulation has been found in another study where the authors suggested an explanation that the CtsR repressor needs ClpC protein to be active (Chastanet et al., 2003). Also, mycolactone induced

metabolic flux, which may influence the availability of intracellular ATP levels required for repression, might also have an impact on the transcriptional control of both CtsR- and HrcA operons (Chastanet et al., 2003).

Several genes involved in stress response, including both copies of the cold shock protein *cspC* and *csbD*, a sigma B controlled gene product involved in general stress response, were downregulated (Chaves-Moreno et al., 2016). Genes for signal transduction and transport including *sarR*, a LysM peptidoglycan-binding domain-containing protein were also downregulated.

Our RNASeq data also showed *hla*, *agrA*, and *SaeR* genes were modulated across timepoints (Supplemental Table 1). However, these data were not statistically significant. And while these RNASeq data give plausible explanation for transcriptome differences between *S. aureus* wild-type and mycolactone supplemented treatments, it will be necessary to repeat experimental work with increased replication to elucidate these processes in more detail. Future work will also include analyses of the *S. aureus* secretome during mycolactone supplementation.

Presence of other bacteria such as *S. aureus*, and other pathogens with virulence potential, isolated from BU wounds could contribute to the delay in wound healing of patients, especially following the initiation of antibiotic treatment against *M. ulcerans* (Amissah et al., 2017). However, without further studies on *S. aureus* virulence factor expression and other microorganisms, within or isolated from BU wounds, as well as host response within this context, the role of *S. aureus* in delaying wound healing has only been presumptive. Within the wound, it is therefore important to understand the interaction of *M. ulcerans* with skin flora and other wound residents in determining *M. ulcerans* infection and pathology, as well as to measure local immune response to co-infection. This is also important in determining treatment outcomes of BU

following antibiotic treatment where *M. ulcerans* replication in is slowed or ceased (World Health Organization, 2012a). Only three isolation studies have been conducted on *S. aureus* contamination in BU wounds, but have reported *S. aureus* in 14.5% to 63.3% of BU wounds (Amissah et al., 2015; Anyim et al., 2016; Yeboah-Manu et al., 2013), indicating an urgent need to understand these interactions.

Amissah et al. (2015) isolated *S. aureus* from 30 BU patients. From these, 26% of the isolates showed the same *S. aureus* genotype from individual patients' wound and nose, but with *agr*-type diversity; many of these isolates were also resistant to clinically relevant antibiotics (Amissah et al., 2015). *S. aureus* colonizing BU wounds belonged to *agr* types II, III and IV, which are responsible for diseases such as toxic shock syndrome and Staphylococcal Scalded Skin Syndrome due to production of TSST-1 and exfoliatin respectively (Amissah et al., 2017; Bibalan, Shakeri, Javid, Ghaemi, & Ghaemi, 2014) . A recent study of 21 of those *S. aureus* isolates showed temporal changes in *S. aureus* genotypes in the wounds of some BU patients, with some *S. aureus* genotypes containing additional virulence genes over time, though all harbored core virulence genes (Amissah et al., 2017). It was also interesting in that study, that, in many of the *S. aureus* isolates, α-, β- and δ-hemolysin genes were present, though their activity was only detected in some isolates (Amissah et al., 2017). It was hypothesized that the lack of hemolytic activity could be due to a suppression of *agr* function by upstream regulators, such as *sigB;* however, this was not assessed in that study (Amissah et al., 2017).

Data presented in our study show that mycolactone downregulates *S. aureus* global regulators and QS machinery in a dose dependent manner. Further, mycolactone attenuates *S. aureus* virulence without inhibiting *S. aureus* growth and suppresses *hla* promoter activity and hemolysis. These data are relevant and significant as mycolactone and *S. aureus* sensing and

response at the transcriptional, translational and regulation levels will provide insight into biological mechanisms of interspecific interactions that may play a role in regulation of responses such as effects between MU, mycolactone, and *S. aureus* virulence, gene expression, proteome and exometabolome, toxin production, and MU-specific QS antagonism. These data have implications for microbial interactions with *M. ulcerans* and other microbes, and mycolactone mechanisms for *M. ulcerans* fitness in natural environment and host niches. For instance, it is well known that changes in microbiomes can promote resistance to or infection by pathogenic bacteria. Our study also describes how a pathogen can modulate regulatory signals derived from skin microbiota (normal flora and those with pathogenic potential), further defining interspecies interactions. Results of these data suggest that BU pathology may, in part, be the result of multispecies synergistic and antagonistic interactions, further increasing disease complexity. More broadly, acute and chronic wound infections are a significant health problem around the world. And, data evaluating the roles of microorganisms and their specific interactions, as well as host immune response in this context, will have consequences for understanding disease pathogenesis, wound healing, and better patient management, as this knowledge is critical for successful management of wounds.

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CONCLUSIONS

Buruli ulcer disease (BUD) remains a 'mysterious disease' due to the unknown mode of *M. ulcerans* transmission and pathogenesis (Roltgen, K and Pluschke, 2015). To understand these, it is important to determine the reservoir of the organism in its natural environments, and stress response and interactions of *M. ulcerans* in its natural niche and during infection to a host. The major virulence factor of *M. ulcerans* is mycolactone, a lipid cytotoxin that is encoded on a giant plasmid pMUM001 (Sarfo et al., 2016). It is suggested that plasmid pMUM001 was acquired by *M. ulcerans* during evolution from its progenitor, *M. marinum* (Yip et al., 2007). According to the coincidental evolution of virulence hypothesis, many microbes evolve to acquire traits to outcompete or overcome biotic and abiotic forces during their normal life cycle in the outside-host environment. However, these traits, such as toxin production, could confer virulence during infection to hosts. Thus, the primary function of these virulence factors is to provide a fitness advantage in the environment. This study was focused on investigating stress response of *M. ulcerans* against selective abiotic and biotic forces and the role of mycolactone in determining the polymicrobial interaction in its natural environment and under abiotic or biotic conditions that may be encountered during infection to human hosts.

Although an exact mode of transmission for BUD is unknown, there are various postulated modes of transmission that include through passive transmission via cuts and wounds during contact with environments containing *M. ulcerans*, bites of aquatic insects or mosquitoes

or transmission via amoebae (Lavender et al., 2011; Marion et al., 2010; Wilson et al., 2011). Failure to establish a passive infection in abrasion sites in an animal model raised questions regarding transmission, such as whether puncture of *M. ulcerans* contaminated skin will lead to disease pathology, whether the skin microbiome affects infection, and whether mycolactone is modulated differentially with response to differing routes of infection (for instance, abrasion versus injection) (Williamson et al., 2014). Additionally, natural environments consist of several abiotic and biotic stresses such as sunlight-UV, salinity, pH, nutritional stresses, etc, which can play role in determining the reservoir of bacteria. Chapter I of this dissertation consists of a comprehensive introduction of Buruli ulcer disease. In this chapter, we provided information on various aspects of the disease such as its etiological agent, clinical presentation, diagnosis, prevention and treatment, socio-cultural, psychological and economic impact of the disease along with pathology and virulence of *M. ulcerans*. We also discussed previous and ongoing researches, challenges and provided future directions needed to understand mode(s) of transmission of BUD. This chapter also supported a One-Health objective to establish a synergistic global network of qualified individuals working locally, regionally, nationally, and internationally to provide the comprehensive understanding of the complex interdependences of human, animal, microbial and ecosystem health influencing transmission of *M. ulcerans* and prevention of BUD.

Chapter II of this dissertation introduced toxin producing prokaryotic and eukaryotic environmental pathogens. It provided a review on functions of several toxins produced by environmental pathogens in providing a fitness advantage to the microbe in their natural, environmental life cycle. It also illustrated how these roles of toxins, such as nutrient acquisition,

adherence to host, and protecting against biotic and abiotic stresses and predation in the environment could enhance survival, attachment, colonization, and invasion during infection to human host. Hence, this chapter supported the coincidental evolution of virulence hypothesis and suggested that microbes adapt to acquire novel weapons such as toxins in response to several abiotic and biotic forces in the environment. However, these novel weapons can confer virulence upon incidental infection to host.

Chapter III of this dissertation provided significance of conducting the overall outlined research.

Chapter IV of this dissertation investigated the effects of UV radiation, higher than optimal temperature, lower than optimal oxygen conditions and nutritional stress on *M. ulcerans* growth and global, and mycolactone gene expression. The results from this chapter showed that exposure of *M. ulcerans* to abiotic stresses such as UV, higher temperature and lower than optimal oxygen conditions modulate its global and mycolactone gene expression. These findings are important as it hints toward potential reservoirs or environmental niches, and pathogenesis of *M. ulcerans.* The upregulation of mycolactone under microaerophilic conditions suggested that *M. ulcerans* may reside in a microaerophilic environment and mycolactone may provide a fitness advantage in those conditions. There was no significant effect of UV on *M. ulcerans* growth which suggests that *M. ulcerans* may have higher tolerance to UV than previously thought and mycolactone may not play role to provide fitness advantage against it. Further, exposure of *M. ulcerans* to a combined higher temperature and lower than optimal oxygen condition, which are

environmental conditions that *M. ulcerans* could be experiencing during infection to human hosts, upregulated several stress response genes. These genes may be evolutionary conserved stress mechanisms of *M. ulcerans* in its natural habitat, however, it may enhance survival and virulence upon entry to human hosts. Further, exposure to combined high temperature and hypoxic environments downregulated mycolactone expression. During infection, *M. ulcerans* multiplies intracellularly but later lyse the cell and becomes extracellular (Torrado et al., 2007). It is suggested that mycolactone is turned off during this initial intracellular growth (Torrado et al., 2007). The intracellular environment of phagocytes have higher temperature (37^0C) and lower oxygen concentrations than that of the extracellular environment (Healer, Graszynski, & Riley, 1999; James, Grinberg, Michaels, & Swartz, 1995). Hence, findings from our study provide clues to this observation and suggest that higher temperature and hypoxic environments could be some of the factors triggering the initial turn off of mycolactone.

Chapter V and VI were focused on understanding the role of mycolactone in polymicrobial interactions of *M. ulcerans* in its natural habitat and under potential biotic conditions during infection to human hosts, respectively. Results from chapter V showed that mycolactone can impact overall microbial community structure in a polymicrobial environment in its natural, aquatic habitat. Mycolactone interacted selectively at the Phylum level as there was a positive relationship with Firmicutes whereas negative relationships were identified with Actinobacteria. Further, mycolactone was associated with increased relative abundance of some families such as Comamonadaceae, whereas mycolactone was associated with decreased relative abundance with Sphingomonadaceae and Intrasporangiaceae. Although there were no clear differences in general characteristics of overall microbial community structure based on their

interaction with mycolactone, other factors related to microbes such as their cell-wall component and individual response to quorum sensing or quenching signals could have impacted their affinity to mycolactone. Indeed, many Families identified are known to utilize quorum sensing and quorum quenching activity for phenotypic and genotypic response to abiotic, biotic, and antibiotic stressors.

Results from chapter VI showed that mycolactone affects virulence and quorum sensing in an opportunistic pathogen, *S. aureus,* without inhibiting its growth. In this study, mycolactone downregulated the expression of genes in the operon and two component system of global regulators Agr and SaeR, respectively, in a dose dependent manner. Further, mycolactone attenuated *S. aureus* virulence by suppressing *hla* promoter activity that downregulated *hla* gene expression and hemolysis. RNA seq analysis showed that mycolactone limits oxygen supply and suppresses *S. aureus* translocation and secretion system as genes related to these functions (oxidase, secY and *flo*A) were downregulated in thw presence of mycolactone. Moreover, mycolactone modulated expression of genes related to metabolism of amino acids, carbohydrates, nucleotides, energy and lipids thereby impacting every facet of *S. aureus* metabolism. Similarly, several stress response genes such as cell wall stress stimulon (*clpC*, *clpB*, and *clpX*) and heat shock proteins (*GroE*L, *GroES*, *DnaK*, and *DnaJ*) were upregulated in the presence of mycolactone, indicating damage and denaturation of cell-wall proteins.

Mycolactone is structurally similar to homoserine lactones and has been shown to continuously increase after bacterial cultivation reached a plateau in an animal model, suggesting that mycolactone may be used as a quorum sensing mechanism(Fred Stephen Sarfo et al., 2013). Further, macrolides such as erythromycin, clarithromycin and azithromycin have been shown to

exhibit quorum quenching effects against *P. aeruginosa*, which suggests a potential role of other macrolides, such as mycolactone, as quorum quencher(Tateda et al., 2007). Quorum quenching molecules such as dextranase and proteases produced by *Arthrobacter* and *Kribbella* (family Nocardioidaceae), respectively, can inhibit biofilm formation in gram positive bacteria such as *Streptococcus mutans* and *Staphylococcus aureus*(Sarveswari & Solomon, 2019). The ability of *M. ulcerans* to utilize mycolactone in quorum sensing or quorum quenching activity could allow *M. ulcerans* to sequester signals, or suppress gene expression in competing microbes, or facilitate antagonistic activities. Furthermore, the understanding that *M. ulcerans* can impact quorum sensing mechanisms also suggests the possibility that *M. ulcerans* can also eavesdrop on other microbial signals to facilitate responses to nutrient limitation, other abiotic conditions, and host infection and immune response.

Data presented in Chapter VI is the first study to investigate the quorum quenching ability of mycolactone and its potential role in determining polymicrobial interactions of *M. ulcerans.* These findings are important as it will provide better insight of the role of mycolactone as a quorum sensing or quorum quenching molecule to survive social life in its natural habitat and during host infection, where other microbes, such as *S. aureus* and *P. aeruginosa* have been isolated(Amissah et al., 2015; Barogui et al., 2013; Yeboah-Manu et al., 2013).. Being that *M. ulcerans* leads to a necrotizing skin infection upon subdermal inoculation, understanding the interaction of *M. ulcerans* with skin flora will also aid in determining how interactions with skin microbes may impact *M. ulcerans* infection. Further, studies on the effects of mycolactone on secondary colonizers of BUD such as *S. aureus* is important in determining treatment outcomes of BUD following antibiotic treatment, and also mechanisms leading to delayed wound healing.

Currently, mycolactone research is focused primarily on mycolactone effects on human hosts, with little attention paid to the role of mycolactone in environments external to hosts. Though those studies provide insight from a clinical perspective of disease understanding, the bacterial perspective is also necessary to understand factors triggering bacterial genes and molecules leading to increased virulence and disease pathogenesis. This dissertation work moved beyond the status quo by focusing on bacterial gene modulation in response to abiotic and biotic environmental parameters, which can guide preventive and treatment strategies for human diseases. Further, our novel research of synergistic or antagonistic interactions within the complex polymicrobial communities colonizing skin and aquatic habitats is a powerful approach in determining *M. ulcerans* colonization efficiency and resiliency and transmission mechanisms.

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APPENDIX A

APPENDIX TABLE

Table A.1 Review of papers investigating presence of *M. ulcerans* in the environment (Methods and *M. ulcerans* positivity)

Methods for *M. ulcerans* detection	Further analysis to confirm *M. ulcerans* positivity	*M. ulcerans* presence	BU endemic and non-endemic locations results	References
quantitative PCR- IS2404-Kr		yes		Garchitorena et al. (2015)
semi-quantitative PCR - IS2404, Quantitative PCR- ER		yes	higher MU positivity in well-filtrand collected in endemic location compared to non-endemic locations	Williamson *et al.* (2012)
Real time PCR IS2404-IS2606-KR		yes		Maman *et al.* (2018)
Real time PCR- IS2404-KR-B		yes	significantly higher MU positivity in endemic locations compared to non-endemic locations	Tian *et al.* (2016)
Real time PCR-IS2404 - IS2606-KR	VNTR-sequenced - database searches of GenBank were performed using the BLASTN algorithm.	yes		Fyfe *et al.* (2007)
Real time PCR-IS2404 and KR		yes		Morris *et al.* (2014)
Real time PCR-IS2404-IS2606-KR		yes		Bratschi *et al.* (2014)
Real time PCR-IS2404-IS2606-KR, IS2404/IS2606 copy number ratio		yes		Aboagye *et al.* (2017)
Real time quantitative PCR-IS2404-KR		yes		Combe *et al.* (2019)
Real-time PCR- IS2404-IS2606-KR		Too low		Vandelannoote *et al.* (2010)
real-time PCR- IS2404-IS2606-KR		yes	higher MU positivity in BU endemic location compared to non-endemic locations	Hammoudi *et al.* (2020)
Conventional PCR- 16S rRNA- IS2404- ER	VNTR typing-sequenced using (Sanger sequencing)-NCBI BLAST	Detected MPMs		Dassi *et al.* (2017)
nested PCR IS2404- PCR	Sequenced and BLASTn seaarched	IS2404 positivity		Hennigan *et al.* (2013)
PCR-IS2404, BACTEC culture	Mice inoculation by PCR positive culture and observed for virulence. Culture and PCR performed from developed lesion	yes		Marsollier *et al.* (2004)

Table A.1 (continued)

Methods for *M. ulcerans* detection	Further analysis to confirm *M. ulcerans* positivity	*M. ulcerans* presence	BU endemic and non-endemic locations results	References
PCR-ER		yes	difference seen between endemic and non-endemic locations	McIntosh *et al.* (2014)
PCR- IS2404-16S rRNA, Cultured on LJ medium	Identified based on phenotypic characteristics, and sequencing of 16S rRNA	no IS2404 positivity		Eddyani *et al.* (2008)
PCR-16S rRNA- IS2404-ER, Cultured on LJ media	VNTR-Sequenced randomized samples (Multi sequence alignments (MSA) and phylogenetic analyses were performed within MEGA V5)	yes		Narh *et al.* (2015)
PCR-IS2404-ER	VNTR	yes	no M. ulcerans in non-endemic locations	Pileggi *et al.* (2017)
PCR-heat shock protein 65, Cultured on LJ medium supplemented with INH, PM medium, or DF medium	sequenced and BLAST search, *M. ulcerans* isolate obtained- confirmed using PCR-IS2404-IS2606- KR-rpoB	yes		Samuel *et al.* (2016)
PCR-IS2404, IS2606- 16S rRNA, Culture using IMS method	southern blot hybridization analysis	yes		Stinear *et al.* (2000)
PCR-16S rRNA-IS2404-ER	VNTR	MPMs detected		Tano *et al.* (2017)
PCR-IS2404-ER-VNTR	VNTR	yes	Human endemicity did not predict MU prevalence in Ghana (may be because of passive surveillance)	Williamson *et al.* (2008)

Table A.2 General characteristics of the most important taxa and their relationship with mycolactone

Family	Interaction with mycolactone	Phylum	GRAM	Temperature requirement	Aerotolerance	Motility
Comamonadaceae	+	Proteobacteria (Beta)	-	Mostly mesophilic	Aerobic or anaerobic	Motile
Xanthomonadaceae	+	Proteobacteria (Gamma)	-	Mesophilic	Aerobic or strictly aerobic	Mostly motile
Moraxellaceae	+	Proteobacteria (Gamma)	-	Mostly mesophilic (*Psychrobacter*-pshychrophilic)	Mostly strictly aerobes or aerobes	Mostly non-motile
Clostridiaceae	+	Firmicutes	+	Mostly mesophilic	Obligate anaerobic	Motile or non-motile
Bacillaceae	+	Firmicutes	+	Mesophilic, psychrotrophic, and thermophilic	Aerobic or facultatively anaerobic	Mostly motile
Flavobacteriaceae	+	Bacteroidetes	-	Mostly mesophilic	Mostly aerobic	Non-motile or Exhibit gliding motility

Table A.2 (continued)

Family	Interaction with mycolactone	Phylum	GRAM	Temperature requirement	Aerotolerance	Motility
Sphingomonadaceae	-	Proteobacteria (Alpha)	-	Mostly mesophilic	Aerobic or partially facultative anaerobic	Motile or non-motile
Bradyrhizobiaceae	-	Proteobacteria (Alpha)	-	Mesophilic	Aerobic	Mostly motile
Intrasporangiaceae	-	Actinobacteria	+	Mesophilic	Aerobic or facultatively anaerobic	Mostly non-motile
Nocardioidaceae	-	Actinobacteria	+	Mostly mesophilic	Aerobic	Motile
Micrococcaceae	-	Actinobacteria	+	Mostly mesophilic	Aerobic	Non-motile
Burkholderiaceae	+/-	Proteobacteria (Beta)	-	Mostly mesophilic	Mostly aerobic	Mostly motile
Caulobacteraceae	+/-	Proteobacteria (Alpha)	-	Mostly mesophilic	Aerobic, or facultatively anaerobic	Motile
Phyllobacteriaceae	+/-	Proteobacteria (Alpha)	-	Mesophilic	Aerobic	Motile
Chitinophagaceae	+/-	Bacteroidetes	-	Mesophilic	Aerobic or facultative anaerobic	Mostly non-motile

Table A.3 Comparison of Beta diversity (Adonis) between different locations and treatment groups

Comparison groups	df	F-model	R^2	P-value
Endemic vs. Non-endemic	1	1.1301	0.27363	0.4
Mycolactone vs. control slides	1	0.70771	0.05569	0.753
Mycolactone vs. control slides (in endemic location)	1	0.817	0.08322	0.62
Mycolactone vs. control slides (in non-endemic location)	1	2.7136	0.73072	0.33

Table A.4 Results on P-value for Chi-square test comparing general characteristics of top 10 most important families based on their affinity to mycolactone

General characteristics	Gram status	Temperature requirement	Oxygen Requirement	Motility
P-value	0.165	0.64	0.642	0.63

APPENDIX B

APPENDIX FIGURES

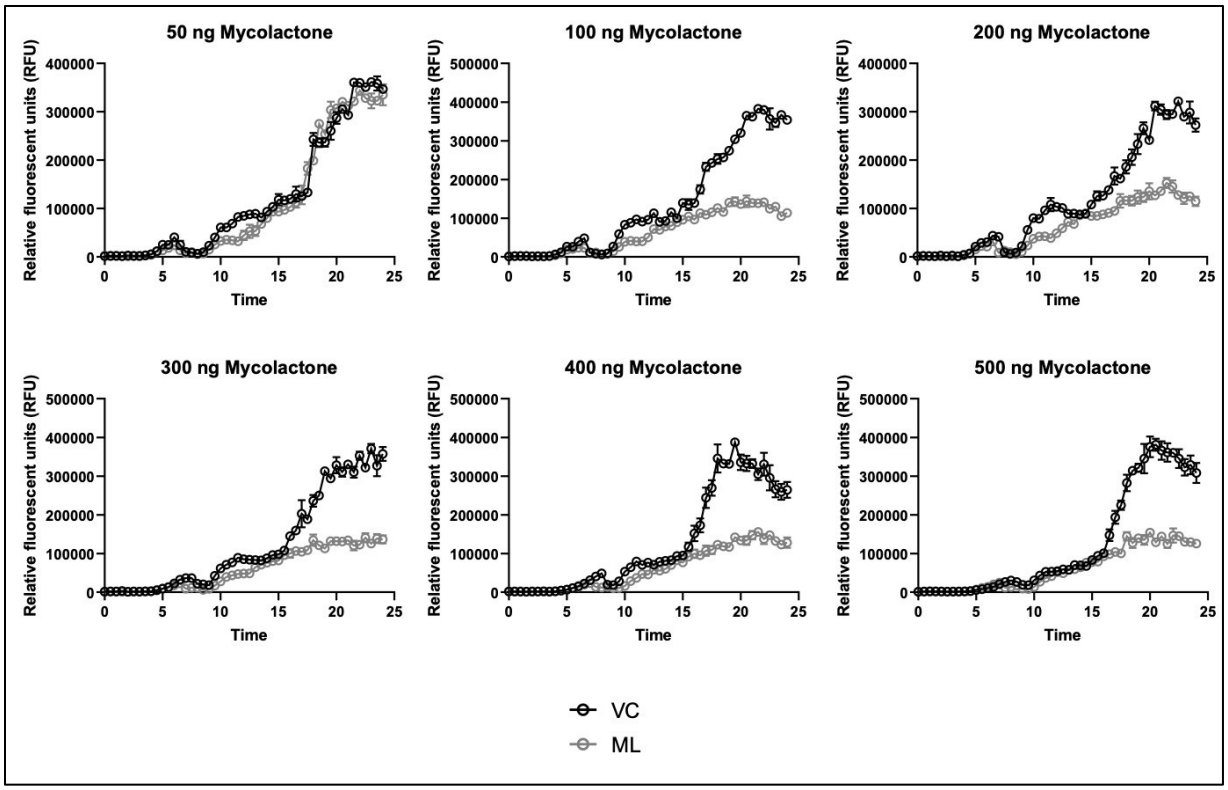

Figure B.1 Promoter activity of *hla* in culture supplemented with mycolactone (ML, 200-500 ng) or vehicle control (VC, EtOH) measured by a luminescent reporter gene.

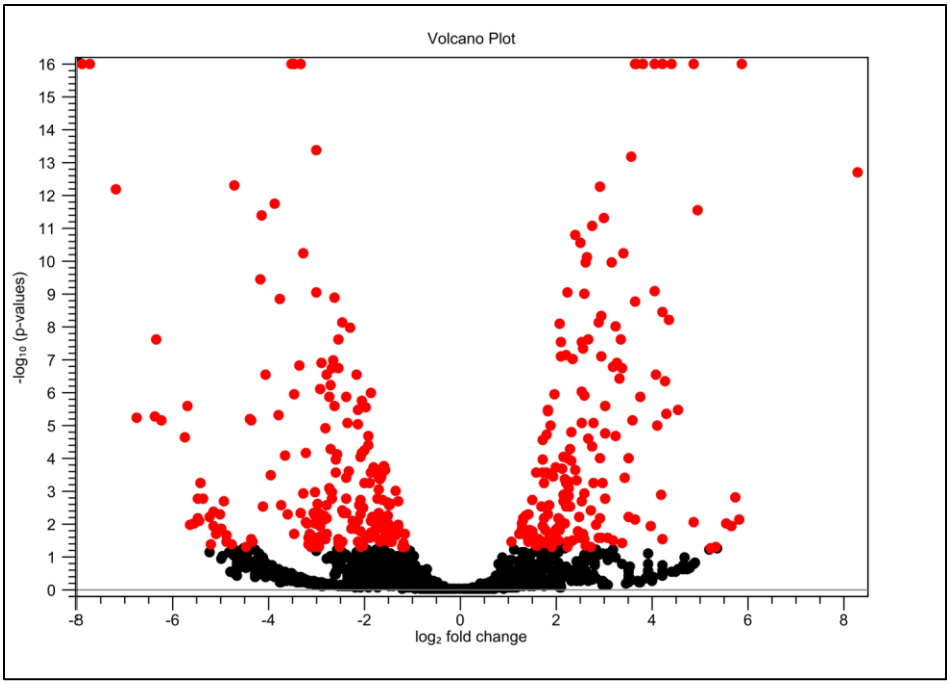

Figure B.2 Volcano plot showing statistical significance (-$\log_{10}$ p-value) versus magnitude of transcription change ($\log_2$ fold change) in *S. aureus* + EtOH versus *S. aureus* + mycolactone at 3H, 6H, and 24H. Statistically significant differences are shown in red.