Microbiology of *Mycobacterium Ulcerans*

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40.1 Properties of *M. ulcerans*

Mycobacterium ulcerans, the etiologic agent of Buruli ulcer (BU), is a slowly growing acid-fast bacillus, growing optimally at 30° to 33°C on common mycobacteriologic media such as Löwenstein-Jensen medium. The organism is microaerophilic, often requiring 6- to 12-week incubation to achieve isolation in primary culture. Subcultures are generally positive within 3–4 weeks of incubation. Colonies suggestive of *M. ulcerans* appear yellowish and rough and have well-demarcated edges. African and Japanese strains are more yellowish than Australian strains, which are cream in color.

Tests for phenotypic identification were described elsewhere [1], but, presently, the identification of *M. ulcerans* is done by genetic tests (see Chap. 41).

MacCallum and associates were the first to isolate *M. ulcerans* in culture in 1948 from a patient in Australia [2].

The epidemiology of BU is strongly associated with wetlands, especially with slow-flowing or stagnant water bodies which implicates that the source of the organism is related to the environment. After a multitude of attempts to cultivate the organism from the environment over half a century, the first cultivation of *M. ulcerans* from an aquatic environment (from a water strider) was reported in 2008 [3].

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40.2 Genetic Diversity of *M. ulcerans*

40.2.1 Characteristic Features of *M. ulcerans* Genome

The genome of *M. ulcerans* strain Agy99 isolated from a BU patient from the Amansie West district of Ghana was the first to be fully sequenced and published. It comprises two replicons, a 5.6 Mbp circular chromosome and a circular 174 kpb giant plasmid (pMUM001) [4]. The chromosome contains 4160 protein coding sequences (CDS) and has a G+C content of 65%, large numbers of insertion sequence elements (ISEs) (209 copies of IS*2404* and 83 copies of IS*2606*), and two bacteriophages phiMU01 (18 kb, 18 CDS) and phiMU02 (24 kb, 17 CDS). There are 45 genes encoding tRNA and a single rRNA operon [4].

The plasmid contains three large genes (*mlsA1*, 51 kb; *mlsA2*, 7.2 kb; *mlsB*, 43 kb) that encode the polyketide synthases (PKSs) required for the synthesis of mycolactone, the primary virulence factor of *M. ulcerans* [5]. The plasmid also contains four copies of the insertion sequence IS*2404* and eight copies of IS*2606* [6].

M. ulcerans and *M. marinum* are genetically closely related as they share more than 4000 orthologous and syntenic CDS and have an average sequence identity of 98.3% [4, 7]. Comparative genome analysis of *M. ulcerans* Agy99 with the clinical *M. marinum* strain M gives indication that pMUM001 and ISEs represent the main differences between the two genomes and that acquisition of these elements is a landmark in the evolution of *M. ulcerans* from a *M. marinum* progenitor that acquired the elements through horizontal transfer [4, 8].

Although *M. ulcerans* has acquired foreign genetic elements, its genome has diminished in size compared to *M. marinum* as a result of multiple rearrangements and deletions of large sections of the chromosome mainly brought about by the transposition activities of the ISEs. Thus there is about 1046 kb of DNA deleted from *M. ulcerans* genome that are present in *M. marinum* [4, 9]. In all there are 157 regions in *M. marinum* that are absent from *M. ulcerans*. These regions form *M. ulcerans* regions of difference (MURDs). Notable MURDs include secondary metabolism, intermediary and energy metabolism, and PE/PPE genes [4].

Another remarkable feature of the genome of *M. ulcerans* is the accumulation of pseudogenes, and it is estimated that about a quarter of the predicted ancestral protein coding genes have undergone mutation and are therefore inactive [4]. These mutations have resulted in the loss of many virulence factors and immunogens in *M. ulcerans* compared to *M. marinum*. Prominent among which is the drastic reduction in cell surface proteins PE and PPE. Compared to *M. marinum* which has 170 PE and 105 PPE, *M. ulcerans* has only 70 PE and 46 PPE genes. This accounts for 45% of the MURDs. A total of 111 of the predicted pseudogenes of *M. ulcerans* were created by insertion of ISEs [4]. The reduction in the PE/PPE proteins has resulted in the depletion of the related ESX secretion systems and their effector proteins. The ESX loci encode type VII secretion systems [10] and are required to export members of the ESAT-6 (6 kDa early secretory antigenic target) protein family and specific effectors, such as EspA (ESX-1 secretion-associated protein A).

While the genome of *M. marinum* contains two copies of the esxB–esxA gene cassette, members of the *M. ulcerans* ancestral lineage (isolates of patients from the Americas and Asia) have retained only one copy of this gene cluster. Both copies are deleted from the genome of *M. ulcerans* strains belonging to the classical lineage (isolates of patients from Africa, Papua New Guinea, and Australia) [11]. The resulting reduction in abundance or complete loss of strong B- and T-cell immunogens may help *M. ulcerans* to evade host immune responses and may confer a survival advantage in host environments that are screened by immunological defense mechanisms [11].

In the same vein, *M. ulcerans* has also lost capacity to produce phenolic glycolipids (PGL), cell wall components, antigens, and major virulence factors for several mycobacterial pathogens that can also modulate innate immunity. *M. marinum* synthesizes PGL through glycosylation of phenolphthiodiolone, a polyketide-derived intermediate. *M. ulcerans* also produces phenolphthiodiolone but cannot make PGL as a result of inactivation of the glycosyl transferase genes involved in the synthesis process [4, 12].

M. marinum has the *crtI* locus that encodes for the production of phytoene dehydrogenase, an essential enzyme for the biosynthesis of light-inducible carotenoid pigments which protects the bacterium from damage following exposure to sunlight [13]. Although, the *crtI* gene is also present in *M. ulcerans*, it is inactive due to the insertion of a premature stop codon in the gene, suggesting that the pigments are not required for survival, presumably because *M. ulcerans* inhabits dark environments and is not exposed to sunlight [4].

The complements of genes encoding enzymes involved in aerobic respiration are preserved in both *M. ulcerans* and *M. marinum*, and both organisms are thus capable of growth under aerobic conditions. *M. marinum* however retains capacity for anaerobic respiration utilizing pathways that involve the coupling of fumarate oxidation with nitrite reduction or through nitrite reduction via the *nirBD* nitrite reductase and NarK transporter. *M. ulcerans*, on the other hand, has lost the fumarase dehydrogenase system as well as the *nirB* and *narK* loci (which are pseudogenes) and consequently cannot undergo anaerobic respiration [4].

Comparative whole genome analysis has revealed that *M. ulcerans* evolved from a *M. marinum* progenitor through acquisition by lateral transfer of a virulence plasmid and ISEs. Transposition activities of ISEs have resulted in deletions of large segments of the chromosomes, rearrangements in the genome, inactivation of genes, and accumulation of pseudogenes [4, 7]. Thus, ISEs are important in leading the genome reduction drive in *M. ulcerans*. Analysis of isolates also has revealed a high level of genetic homogeneity in *M. ulcerans* leading to a clonal population structure for this pathogen [14].

These features are characteristic of bacterial populations that have passed through an evolutionary bottleneck suggesting there has been constriction of population size during adaptation to a new niche environment [15, 16]. Although the niche of *M. ulcerans* is yet to be determined, the loss of genes expressing potent T-cell antigens and genes required for pigment synthesis, anaerobiosis, and intracellular growth suggests that the bacterium has adapted to a dark, extracellular environment

where slow growth rate, loss of immunogens, and production of an immunosuppressive molecule provide a selective advantage [4].

By providing clues on the environmental niche of *M. ulcerans*, whole genome comparison can aid in the development of targeted and appropriate measures for the primary prevention of BU. Comparative genomics can also potentially lead to the identification of new targets for development of rapid diagnostic tests to augment early detection and treatment of cases, which is the current strategy for BU control.

40.2.2 Strain Typing and Molecular Epidemiology

The high level of genetic homogeneity among *M. ulcerans* isolates is a major impediment to unravelling the environmental reservoir as well as the route through which *M. ulcerans* infects humans and animals [17]. For several decades, investigators have applied high discriminatory genotyping techniques including VNTR [17–19], RFLP [20], IS*2404*- Mtb2-PCR [21], *2426* PCR [22], and MLST [14] to uncover genetic diversity in *M. ulcerans* for molecular epidemiology investigation of BU. However, these methods only resolved *M. ulcerans* on the basis of their continent of origin with no or limited genetic differences on continent and country basis. The level of clonality was highest among African isolates [17–22].

Inspired by the high resolution afforded by single nucleotide polymorphism (SNP) typing, a set of 65 SNP loci was used to investigate the phylogeography and transmission pathways of *M. ulcerans* in endemic communities of the Densu river basin in Ghana [23]. The study identified ten *M. ulcerans* haplotypes with a particular type (founder haplotype) distributed widely across all the endemic communities studied, while the other haplotypes formed local clonal complexes that were confined to individual endemic foci with no evidence of mixing of haplotypes (Fig. 40.1). Comparison of SNP profiles with those of neighboring and distant isolates showed that the Densu basin haplotypes formed a clade which also comprised an isolate from Togo while the Amansie west isolates clustered together with an isolate from Ivory Coast to form a separate clade. A third clade comprised isolates from Benin, Congo, Angola, and Ivory Coast. Grouping of such a diverse *M. ulcerans* collection has been suggested to represent a phylogenetic bias, a drawback of analysis based on a limited set of SNP loci that may be remedied by expanding the repertoire of SNP loci or interrogating the whole genome [24].

It has now become obvious that for highly monomorphic species such as *M. ulcerans*, comparative whole genome sequence analysis may be the only option available for indexing high level of diversity useful for micro-epidemiological and phylogeographic investigations.

Whole genome sequence comparison approach applied to *M. ulcerans* at the district and village levels in the Cameroon [25] and Ghana [26], respectively, has provided additional information on the nature of distribution of *M. ulcerans* and has also enabled us to form new ideas on how this pathogen could be spreading in communities.

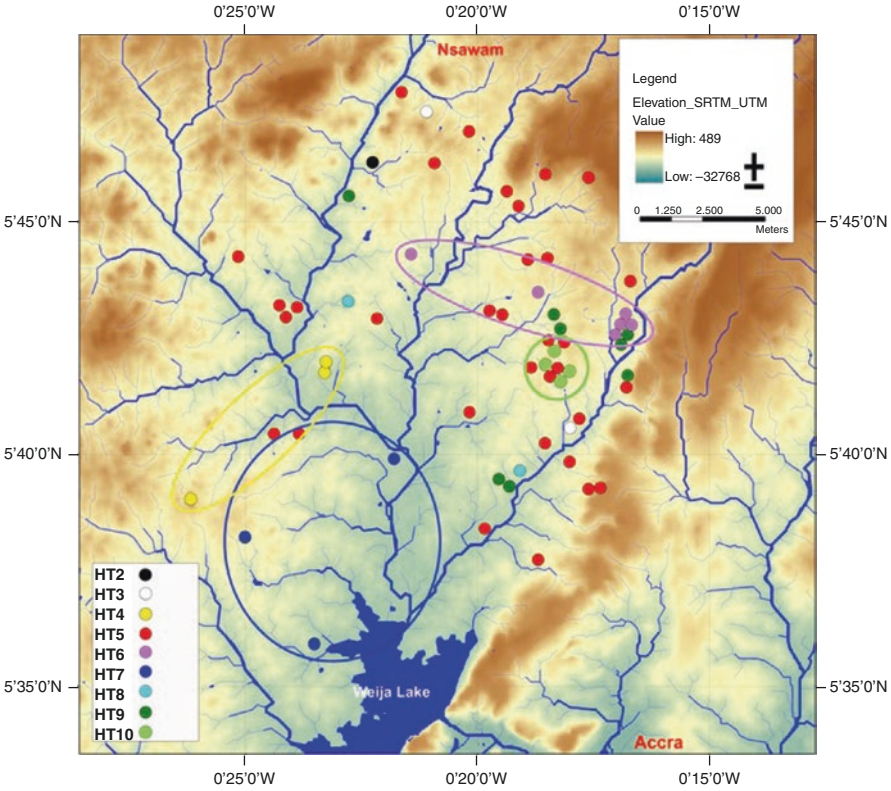
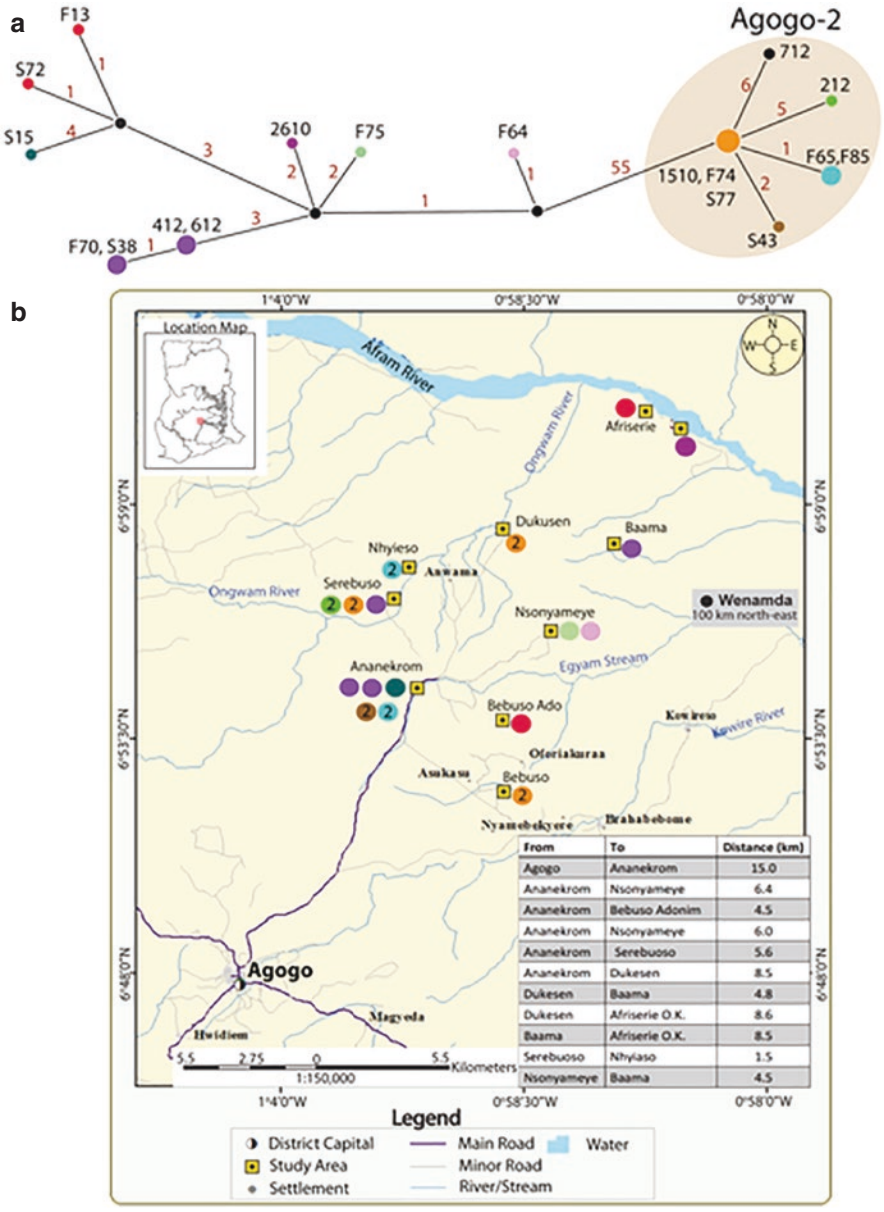


Fig. 40.1 Map of the Densu river basin, showing the homes of patients from whom the strains have been isolated between 2001 and 2006 (colored dots). Haplotypes 2 (*black*), 3 (*white*), 4 (*yellow*), 6 (*purple*), 7 (*dark blue*), 8 (*light blue*), 9 (*dark green*), 10 (*light green*) are unevenly distributed, whereas haplotype 5 (*red*) co-localizes with all other haplotypes. The background map was created using elevation data from the Shuttle Radar Topography Mission (SRTM). Water bodies were classified using optical data from Landsat ETM and radar data from TerraSAR-X

Analysis of the Mape and Nyong river basins in Cameroon uncovered a similar observation as the SNP analysis of the Densu basin where *M. ulcerans* strains form clonal complexes with rare exchanges of strains between distinct endemic areas suggesting focal transmission events occurring within confined endemic foci.

Isolates from Mape, a relatively more recently emerging BU endemic area, were found to be less diverse than populations from longer-standing disease foci of the Nyong basin.

In Ghana, the Asante Akim North study was the first to employ whole genome sequencing to explore the molecular epidemiology of BU at a local scale (clinical isolates from a 30 km^2 region). The study uncovered two *M. ulcerans* clusters, namely, the Agogo 1 and Agogo 2 clusters (Fig. 40.2). The Agogo 1 cluster which comprised closely related isolates from local and neighboring districts of Amansie West and also Ivory Coast represents a local clone that has spread and persisted in



From	To	Distance (km)
Agogo	Ananekrom	15.0
Ananekrom	Nsonyameye	6.4
Ananekrom	Bebuso Adonim	4.5
Ananekrom	Nsonyameye	6.0
Ananekrom	Serebuoso	5.6
Ananekrom	Dukesen	8.5
Dukesen	Baama	4.8
Dukesen	Afriserie O.K.	8.6
Baama	Afriserie O.K.	8.5
Serebuoso	Nhyiaso	1.5
Nsonyameye	Baama	4.5

Fig. 40.2 (**a**) Median-joining network graph showing the genetic relationship between 18 *M. ulcerans* clinical isolates comprising the Agogo-1 and Agogo-2 genotypes (shaded), inferred from whole genome sequence alignments. Node sizes in the graph are proportional to the frequency of genotype occurrence and have been color-coded accordingly. Edges are labelled in red with the number of mutational steps between each node. (**b**) Map of Asante Akim North District study area, showing the location of endemic villages and the origin of each of the 18 BU cases, with a colored circle corresponding with the genotype displayed in the network graph in (**a**). The number "2" within some colored circles indicates an Agogo-2 genotype

the area for some time. The Agogo 2 was more diverse from all other Ghanaian *M. ulcerans* genotypes (138 SNPs) suggesting a likely introduction of isolates from outside Ghana. Further genome comparative analysis identified a strain originating from Ibadan, Nigeria, differing in 29 SNPs from the Agogo 2 cluster, as the closest match among *M. ulcerans* panels assembled from West and Central Africa. The observation of a mixing of local circulating genotypes with isolate clones from distant locations has been reported in Australia [27].

Analysis of the genotype distribution showed for the first time no spatial clustering of genotypes at the local scale with multiple genotypes occurring simultaneously in one village and complete intermixing of Agogo 1 and Agogo 2 clusters among the population (Fig. 40.2). Some patients living in different villages (each separated by distances in excess of 10 kms) were infected with identical *M. ulcerans* genotypes. The suggestion that people moving in the communities become infected from a common point source was deemed unlikely explanation for this observation as further investigations failed to establish epidemiological link between the patients. The possibility of the bacteria or a reservoir spreading it to be widely distributed across the region and infections are being acquired locally seems to be consistent with the whole genome sequence data. The case for local infection is further strengthened by the observation of a 2-year-old infant who had BU but had never travelled outside the village in which she resides.

Comparative whole genome analysis showed for the first time that *M. ulcerans* focal distribution pattern breakdown and multiple *M. ulcerans* genotypes may circulate within a local setting. Multiple genotypes in an area may be the result of accumulated mutations in local clonal complexes over time or the introduction of different genotypes into an area. Also, each *M. ulcerans* genotype may spread equally widely across the region, and the lack of genetic variation among isolates suggests that the spread of *M. ulcerans* throughout a region has occurred relatively rapidly with insufficient time elapsed for mutations to accumulate [26].

It has been suggested that because *M. ulcerans* transmission and microevolution generally occur at a local level, the source of the bacterium is somewhat fixed within a local region, indicating that animal reservoirs of *M. ulcerans* are unlikely to be highly mobile [23].

These whole genome sequence data however give new perspectives on the behavior of possible reservoirs and subsequent transmission mechanisms of *M. ulcerans* and show for the first time that *M. ulcerans* can be mobilized, introduced to a new area, and then spread within a population. Potential reservoirs of *M. ulcerans* thus might include humans or perhaps *M. ulcerans*-infected animals such as livestock that move regularly between countries [26].

40.3 Comments on the Taxonomic Position of *M. ulcerans*

Taxonomy, from the Greek words “taxis” (arrangement) and “nomas” (law), is the science of biological classification. Its purpose is to provide useful ways for identifying and comparing organisms. Classification is the arrangement of organisms into

groups (taxa); nomenclature refers to the assignment of names to taxonomic groups. Throughout the ages, man has given names to living organisms, and that tradition goes back to the very early times, as shown in the very first pages of the Bible. Indeed, at the beginning of the Genesis, we can read that: "The LORD God formed every beast of the field, and every fowl of the air; and brought [them] unto Adam to see what he would call them: and whatsoever Adam called every living creature, that [was] the name thereof" (Genesis, chapter 2, Verse 19).

Once the names are given to "taxa," the characters making it possible to identify them must be described. The choice of these characters is not fixed forever; it can change in the course of time; names too! Taxonomy is thus a dynamic science, in constant evolution.

Two major periods may be distinguished in prokaryotic taxonomy, one characterized by the utilization of phenotypic tests and one characterized by the use of genotypic characteristics. The phenotypic period started at the end of the nineteenth century and the genotypic period during the last decade of the twentieth century and continues to the present.

M. ulcerans can be identified by phenotypic and genotypic tests [1]. In 1997, a specific and sensitive method based on PCR amplification of an insertion sequence, IS*2404*, was developed to identify *M. ulcerans* [28]. Ten years later (in 2007), the genome of *M. ulcerans* was sequenced [4]. In 2012, comparative analysis of whole genome sequences of *M. ulcerans*, other mycolactone-producing mycobacteria (MPM) (*M. pseudoshottsii*, *M. liflandii*, and some *M. marinum* isolates from fish), and *M. marinum* has shown that *M. ulcerans* and all MPM are specialized variants of a common *M. marinum* progenitor and that all MPM differ from *M. marinum* by the acquisition of the pMUM plasmid and introduction of insertion sequences into the chromosome. Based on these findings, it was proposed to consider all MPM *M. ulcerans* ecovars [8]. Although it is correct from a genetic point of view, *M. ulcerans*, *M. pseudoshottsii*, *M. liflandii*, and *M. marinum* have, however, different phenotypic characteristics and, above all, are pathogens of different hosts: *M. ulcerans* is mainly pathogenic to humans and some mammals, while the other MPM are fish and frog pathogens.

Taxonomy should remain pragmatic and clinically meaningful [29]. Although it is sound from a taxonomic point of view, classification of all MPM under a single species should be avoided as it may be very confusing from a medical and epidemiologic point of view.

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