Genotypic and phenotypic diversity of *Mycobacterium tuberculosis* complex genotypes prevalent in West Africa

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Abstract

Findings from previous comparative genomics studies of the *Mycobacterium tuberculosis* complex (MTBC) suggest genomic variation among the genotypes may have phenotypic implications. We investigated the diversity in the phenotypic profiles of the main prevalent MTBC genotypes in West Africa. Thirty-six whole genome sequenced drug susceptible MTBC isolates belonging to lineages 4, 5 and 6 were included in this study. The isolates were phenotypically characterized for urease activity, tween hydrolysis, Thiophen-2-Carboxylic Acid Hydrazide (TCH) susceptibility, nitric oxide production, and growth rate in both liquid (7H9) and solid media (7H11 and Loewenstein–Jensen (L-J)). Lineage 4 isolates showed the highest growth rate in both liquid (p = 0.0003) and on solid (L-J) media supplemented with glycerol (p<0.001) or pyruvate (p = 0.005). L6 isolates optimally utilized pyruvate compared to glycerol (p<0.001), whereas L5 isolates grew similarly on both media (p = 0.05). Lineage 4 isolates showed the lowest average time to positivity (TTP) (p = 0.01; Average TTP: L4 = 15days, L5 = 16.7days, L6 = 29.7days) and the highest logCFU/mL (p = 0.04; average logCFU/mL L4 = 5.9, L5 = 5.0, L6 = 4.4) on 7H11 supplemented with glycerol, but there was no significant difference in growth on 7H11 supplemented with pyruvate (p = 0.23). The highest release of nitrite was recorded for L5 isolates, followed by L4 and L6 isolates. However, the reverse was observed in the urease activity for the lineages. All isolates tested were resistant to TCH except for one L6 isolate. Comparative genomic analyses revealed several mutations that might explain the diverse phenotypic profiles of these isolates. Our findings showed significant phenotypic diversity among the MTBC lineages used for this study.
Introduction

Tuberculosis (TB) remains a global health burden and it is the leading cause of human death from a single infectious agent [1]. TB is caused by members of the Mycobacterium tuberculosis complex (MTBC) which is made up of about 11 genetically highly related subspecies or ecotypes [2]. Human TB is caused mainly by M. africanum and M. tuberculosis; subdivided into nine main phylogenetic lineages (L) which are further regrouped into ‘modern’ and ‘ancient’ lineages [3–8] and exhibit a phylogeographical structure.

A number of studies have now demonstrated the effects of strain diversity among bacterial pathogens [9–13]. These studies highlight the fact that certain strains may cause more invasive diseases than others due to differences in the expression of virulence factors either encoded chromosomally or carried on transmissible elements [14,15]. However, examples of horizontal gene transfer which leads to acquisition of classical pathogenicity islands have not been reported in MTBC, although the MTBC lineages and strains differ phenotypically [13,16,17]. There is now experimental evidence for strain phenotypic diversity in the transmissibility and as well as in the immune response and clinical presentation [18–20]. Some MTBC strains have been associated with large outbreaks, and a propensity to develop drug resistance [21–23]. These observations confirm the need to better understand the diversity in the pathogenesis of the different lineages of MTBC.

Advances in genomics such as whole genome sequencing (WGS) have provided a better understanding of MTBC pathogenesis which includes the identification of essential virulent genes involved in cell surface proteins [24], key enzymes and proteins involved in signal transduction systems [25]. Comparative genomic analyses using WGS have led to the identification of a number of non-synonymous mutations within some of these genes. Based on the observed genomic diversity, some lineages were suspected to be more virulent than others [26,27]. Phenotypic diversity within the MTBC may be attributed to presence of pseudo-genes [28], impaired secretion of virulence-associated proteins [29], and the number of disrupted genes involved in bacterial carbohydrate, lipid and micronutrient metabolism [7,30,31]. However, not all mutations may be translated to phenotypic diversity due to redundancy in specific MTBC pathways [32,33]. Hence, there is a need to investigate the implications of these genomic diversity on the phenotypes expressed by the different lineages.

Most studies on the genotypic and phenotypic characterization of MTBC have concentrated on Mtb lineages with few recent studies on Maf lineages [6,34–37]. However, analysis of the prevalence and spatial distribution of MTBC in Ghana shows that L5 is the most dominant lineage after Lineage 4 (L4) [38]. Ghana is one of the few countries in the world with six out of the 9 phylogenetic lineages of the MTBC causing TB in appreciable numbers and proportions [38]. This makes Ghana a good place to comparatively study the effect of the genetic diversity of the three main MTBC lineages circulating in West Africa (L4 of Mtb and L5 & L6 of Maf) and their phenotypic implications [39]. This study therefore investigated the diversity in the phenotypic profiles of the main prevalent genotypes in West Africa.

Materials and methods

Ethical consideration

Ethical clearance was obtained from the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana (Federal wide assurance number: FWA00001824) and the Ethics Review Committee of the Korle-Bu Teaching Hospital. The details of the study were explained to each participant and written informed consent was obtained from all participants before voluntary enrolment. For participants below 18
years, child assent was sought from the participants and consent from their parents or guardians.

**Mycobacterial isolates**

Isolates used in this study comprised of the three prevalent MTBC lineages in Ghana and West Africa (L4 of Mtb and L5 & L6 of Maf). In total, thirty-six (36) distinct spoligotypes were selected from the Cameroon and Ghana sub-lineages of L4 as well as sub-lineages of L5 and L6 as shown in Table 1. We selected L5 and L6 isolates based on a pre-defined clustering (C) into 5 and three sub-population, respectively [36]. Only isolates susceptible to the two most potent anti-TB drugs, isoniazid and rifampicin and that had been whole genome sequenced [36] were used in this study.

All the mycobacterial isolates were obtained from the sputum of individuals who participated in a previous study [38] and cultured as previously described by Yeboah-Manu et al [40]. The isolates were confirmed as MTBC by PCR amplification of the Insertion Sequence 6110 (IS6110) and genotyped into lineages and sub-lineages using spoligotyping and SNP typing [41]. Susceptibility to isoniazid and rifampicin were determined by the microplate alamar blue cell viability assay [42].

All subsequent phenotypic assays were carried out in duplicates and H37Rv was used as the positive control for the assays.

**Growth assessment**

**Growth assessment on solid media.** Growth assessment on solid media was performed on both Löwenstein-Jensen (L-J) and 7H11 Middlebrook agar (7H11). Löwenstein-Jensen media was supplemented with either glycerol or pyruvate to assess the utilization of different sources of carbon. A loop-full of mycobacteria growing at the log phase was homogenised into cell suspension at McFarland 1.0 for all the assays in this study. The cell suspension was inoculated on the L-J media slants in duplicates for the growth assessment on media slants.

| Lineage(L) | Sub-lineage (s-L) | Growth on 7H11 (TTP&CFU) | Growth on L-J | Growth in Liquid medium | Nitrite | Nitrate | Urease test | Tween hydrolysis | TCH |
|------------|------------------|--------------------------|---------------|-------------------------|--------|--------|------------|----------------|-----|
| L4         | Cameroon         | 1                        | 2             | 3                       | 5      | 2      | 6          | 2              | 6   | 5     | 10        |
|            | Ghana            | 1                        | 2             | 4                       | 4      | 4      | 4          | 4              | 2   | 5     | 5         |
| L5         | L5.C1            | 1                        | 2             | 5                       | 2      | 8      | 2          | 2              | 6   | 9     | 2         |
|            | L5.C2            | 1                        | 1             | 2                       | 2      | 2      | 2          | 2              | 2   | 2     | 2         |
|            | L5.C3            | 1                        | 1             | 1                       | 1      | 1      | 1          | 1              | 1   | 1     | 1         |
|            | L5.C4            | 1                        | 2             | 2                       | 2      | 2      | 2          | 2              | 2   | 2     | 2         |
|            | L5.C5            | 1                        | 1             | 1                       | 1      | 1      | 1          | 1              | 1   | 1     | 1         |
| L6         | L6.C1            | 1                        | 3             | 2                       | 5      | 1      | 6          | 1              | 4   | 3     | 7         |
|            | L6.C2            | 1                        | 2             | 3                       | 2      | 3      | 4          | 2              | 2   | 2     | 2         |
|            | L6.C3            | 1                        | 1             | 2                       | 1      | 1      | 1          | 1              | 2   | 2     | 2         |
| Total number |                 |                          | 10            | 15                      | 20     | 16     | 25         | 25             | 20  | 30    | 30        |

The table summarizes the total numbers of mycobacterial isolates used for each experiment. It displays the number of each lineage as well as sub-lineage.

s-L = sub-lineage, L = lineage.

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was observed every week for 5 weeks and recorded as degree of positivity as previously shown
[43].

Utilization of carbon source was further assessed on 7H11 media plates. All the 7H11
media plates were supplemented with OADC and in addition supplemented with glycerol
(7H11+G) or pyruvate (7H11+P) or both (7H11+G+P) or none (7H11). Homogenized myco-
bacterial isolates at McFarland 1.0 were diluted in 10-fold and 100μL was cultured on the
7H11 plates in triplicates. The time to positivity (TTP) was recorded and the colony forming
unit per mL (CFU/mL) was calculated.

**Growth assessment in liquid medium.** Growth rate was assessed in liquid medium using
7H9 Middlebrook broth (7H9) supplemented with 10% ADC (Difco), 0.05% Tween-80, and
0.2% w/v pyruvate [44]. Approximately 5 mL of the 7H9 medium in 25 mL glass tube were
inoculated with 200 μL of the mycobacterial suspensions at McFarland 1.0. We performed reg-
ular aeration of the tubes under sterile conditions to ensure optimal growth conditions. Absorb-
bance at OD$_{600}$ was measured daily.

**Nitric oxide assay**

Nitric oxide production was determined by measuring the concentrations of nitrate and nitrite
released by the different isolates. We determined the quantity of nitrite and nitrate at the log
phase at McFarland 1.0 using Nitric oxide assay kit (Invitrogen) as previously described [45].
Briefly, this kit uses the enzyme nitrate reductase to convert nitrate to nitrite. Both nitrate and
nitrite were subsequently detected by incubating the reaction mix with a known volume of pre-
mixed Griess reagent. Detection was made as a coloured azo dye product of the Griess reaction
that absorbs visible light at 540 nm. Concentrations of nitrate and nitrite were extrapolated
from a constructed standard curve from standards obtained from the Nitric oxide assay kit.

**Urease activity test**

The mycobacterial suspensions at McFarland 1.0 were inoculated into 25 mL glass tubes with
5 mL of urea broth in duplicates. The set-up was incubated at 37˚C without CO$_2$. The absor-
bance was measured at an OD of 630 nm at 1, 3 and 7 days. A tube with urea broth only was
used as negative control. A colour change in broth from yellow to dark pink or red is indicative
of a positive reaction whereas a negative test had no colour change.

**Tween hydrolysis test**

Tween hydrolysis reagent was prepared by adding 0.5 mL of Tween 80 and 2 mL of a 0.1%
aqueous solution of neutral red to 100 mL of phosphate buffer at pH 7.0. The reagent was then
dispensed in amounts of 5 mL into 16 x 125 mm screw-cap tubes and autoclaved at 121˚C for
10 minutes. A loop-full of mycobacterial isolates growing at the log phase on 7H11 was inocu-
lated into the screw-cap tubes with the tween hydrolysis reagent at McFarland 1.0 in duplic-
ates. The set-up was incubated at 37˚C without CO$_2$. The absorbance was measured at an OD
of 630 nm at 1, 3, 5 and 7 days. A tube inoculated with M. aurum was used as positive control
and a tube with only tween hydrolysis reagent was used as negative control. A colour change
from umber to pink or red was indicative of a positive reaction whilst a negative test retained
the umber colour.

**Thiophen-2-carboxylic acid hydrazide (TCH) test**

We evaluated the drug susceptibility pattern of the selected mycobacterial strains against low
concentration of Thiophen-2-carboxylic acid hydrazide (TCH). Four different batches of
Löwenstein-Jensen media were prepared for this experiment. Two batches were dispensed as drug-free control media with glycerol and pyruvate; to the other two with either glycerol or pyruvate, we added sufficient filter-sterilized TCH to make a final concentration of 2 μg/mL. A 10-fold dilution was made in sterile saline from a culture at a log phase and 10⁻¹ and 10⁻³ dilutions were cultured on the L-J media. A volume of 0.1 mL of each dilution was inoculated on each batch of L-J media. Mycobacterium bovis was used as a positive control. All cultures were incubated for 4 weeks at 37°C and a strain was recorded as resistant to TCH if growth on the drug-containing medium was equal to or greater than 1% of that observed on the drug-free control medium.

Comparative genomic analysis

Whole genome sequence data of the selected isolates were obtained from the European nucleotide archives (accession numbers tabulated in S1 Table). They were mapped unto the H37Rv reference genome (accession number NC_000962) [46,47] using customized bash algorithms communicating to BWA, samtools and bcftools after quality checks with fastqc and trimming using Trimmomatic to obtain the whole genome sequence in fasta format. Specific genes of interest (S2 Table) were extracted using an in-house shell script that communicates to EMBOSS [48] and compared to their respective sister genes in H37Rv for identification of mutations.

Data analysis

Data was analysed and figures were developed by using RStudio Version 1.2.5033 (RStudio, Inc., Boston, MA, USA) with readr, plyr and ggplot2 packages [49–51]. Analysis of Variance (ANOVA) was used to compare the differences across the three lineages of MTBC. In certain circumstances, a two-way ANOVA was used to factor in other variables. Ad hoc testing, tukey test was carried out for analysis that showed significance difference. All analyses were carried out with significance level set at p-value less than 0.05 at 95% confidence level.

All the data for the different lineages in the constructed figures were represented by their standard colours: red = L4, brown = L5 and green = L6.

Results

Growth rate and carbon utilization

Utilization of carbon source on L-J media. We first assessed the utilization of different carbon sources (glycerol and pyruvate) for isolates representative of the three different lineages (Table 1) on L-J media slants. Growth was compared as degree of positivity on glycerol or pyruvate supplemented media slants (Fig 1). Analysis of the average degree of positivity at each time point showed significant difference in growth pattern of the three lineages on glycerol (p<0.001) and on pyruvate (p = 0.005) supplemented media. Further comparison of growth on pyruvate supplemented L-J media showed difference between L6 and L4 (p = 0.003) but not between L5 and L4 (p = 0.2) and L6 versus L5 (p = 0.2). Growth of L4 on glycerol supplemented L-J media over the 5 weeks of observation was significantly higher compared to that on pyruvate (p = 0.004). However, L6 isolates preferred to utilize pyruvate for growth compared to glycerol (p<0.001). Growth of L5 isolates appeared to be similar on both glycerol and pyruvate supplemented L-J media (p = 0.05). This suggests that L5 isolates utilize both glycerol and pyruvate at the same rate for growth.
Growth rate on 7H11

**Time to positivity.** We assessed the growth of the three lineages on 7H11 media plates. The time to positivity (TTP) was measured in terms of the number of days it took for the first colonies to appear for CFU determination (Fig 2). Lineage 4 isolates had the lowest average time to positivity of 15 days (SD = 0) followed by L5 (Average TTP = 16.7 days, SD = 2.9) and L6 (Average TTP = 30.3 days SD = 4.5). There were statistically significant differences between the time to positivity for the three different lineages on 7H11 only media (overall p-value = 0.006; L5-L4 = 0.86, L6-L4 = 0.01, L6-L5 = 0.01).

![Fig 1: Utilization of different carbon sources on Löwenstein-Jensen media slants.](https://doi.org/10.1371/journal.pone.0255433.g001)

**Growth rate on 7H11**

Time to positivity. We assessed the growth of the three lineages on 7H11 media plates. The time to positivity (TTP) was measured in terms of the number of days it took for the first colonies to appear for CFU determination (Fig 2). Lineage 4 isolates had the lowest average time to positivity of 15 days (SD = 0) followed by L5 (Average TTP = 16.7 days, SD = 2.9) and L6 (Average TTP = 30.3 days SD = 4.5). There were statistically significant differences between the time to positivity for the three different lineages on 7H11 only media (overall p-value = 0.006; L5-L4 = 0.86, L6-L4 = 0.01, L6-L5 = 0.01).

![Fig 2: Time to positivity (TTP) on 7H11 media.](https://doi.org/10.1371/journal.pone.0255433.g002)
There was also significant difference between the average TTP of the lineages (Average TTP: L4 = 15.0 days, SD = 0; L5 = 16.7 days, SD = 2.9; L6 = 29.7 days, SD = 5.5) on 7H11+G media (overall p-value = 0.01; L5-L4 = 0.89, L6-L4 = 0.02, L6-L5 = 0.02). There was however no significant difference between the TTP (Average TTP: L4 = 32.5 days, SD = 3.5; L5 = 25 days, SD = 8.7; L6 = 29 days, SD = 5.2) on 7H11+P media (p = 0.5). The average TTP of the lineages (Average TTP: L4 = 35 days, SD = 0; L5 = 31.3 days, SD = 6.4; L6 = 29 days, SD = 5.2) on 7H11+P+G media was similar to that of 7H11+P (p = 0.5).

Determination of CFU. The number of colonies were counted and the CFU per mL was calculated for each mycobacterial isolate. A barplot of logCFU/mL was made for each of the different supplemented media (Fig 3). The logCFU/mL of the lineages was significantly different on 7H11 (overall p-value < 0.001; L5-L4 = 0.2, L6-L4<0.001, L6-L5 = 0.001) and 7H11+G media (overall p-value = 0.04; L5-L4 = 0.18, L6-L4 = 0.04, L6-L5 = 0.34). For growth rate on 7H11, L4 had the highest average logCFU/mL of 6.5 (SD = 0.005) followed by L5 with 6.1 (SD = 0.2) and L6 with 4.3 (SD = 0.3). The same trend was observed on 7H11+G media (Average logCFU/mL: L4 = 5.9, SD = 0.5; L5 = 5.0, SD = 0.6; L6 = 4.4, SD = 0.1). There was however no significant difference among the isolates when growing on 7H11+P (p = 0.23) and 7H11+P +G (p = 0.46).

Growth rate in liquid medium

Since the growth rate of the three lineages on pyruvate supplemented solid media was similar, we compared the growth rate in 7H9 medium with pyruvate as the carbon source. Growth rate was assessed by measuring the optical density at 600nm over a 14-day period at different time points as shown in Fig 4. Again, we showed that there was significant difference in the growth rate of the lineages over the time period of observation (p = 0.0003). Lineage 4 isolates showed the highest average growth rate compared to L5 (p = 0.026) and L6 (p = 0.0002) isolates, respectively. There was, however, no significant difference between isolates of L5 and L6 (p = 0.17). This observation confirms the higher growth rate of L4 isolates compared to the Maf lineages.

Fig 3. Growth rate on 7H11 media. The average logCFU/ml was plotted against the different supplemented media.

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Identification of genotype-specific mutations among genes that may be associated with growth

We compared the amino acid sequence of 22 genes (S2 Table) associated with growth of the MTBC using H37Rv as reference. Three mutations, R16P, Y98H & V75I, in the *ftsE* gene which is associated with cell division were found in L5 and L6 specifically L6.C3, L5.C2 & L5.C1 (Table 2). Also, 2 mutations, R3L and S455A, in the *pstP*, which codes for phosphoserine/threonine phosphatase were found in all L5 and L6 isolates. Again, mutations, A196G and D43A, in *suhB* associated with extragenic suppressor protein, SUHB, were observed in L5 and L6 isolates, respectively. A mutation (E71stop) in *whiB3*, a transcriptional regulatory protein, was found in all L5 isolates. A gene that codes for a probable resuscitation-promoting factor (RPFE), *rpfE*, was also found to have a R126Q mutation in all the isolates and an additional A87V in L5.C1 isolates.

Nitric oxide production and identification of mutations that could be associated with its impaired production

To evaluate the ability of a lineage to produce nitric oxide, we quantified nitrite and nitrate concentrations in culture supernatant. The nitrite concentrations were determined and mean concentrations extrapolated from a standard curve was plotted per lineage (Fig 5). Lineage 5 isolates had the highest mean concentration of nitrite of 5.87 μM (SD = 1.33) followed by L4 (L5 = 4.06 μM, SD = 0.6) and L6 (L6 = 3.91 μM, SD = 0.2) isolates, respectively. We observed significant difference between the nitrite concentrations of L5-L4 (p = 0.01) and L5-L6 (p = 0.01); however, the mean nitrite concentration of L4 was similar to that of L6 (p = 0.96). Mutational analysis of genes which may be involved in nitrite production of L4 showed mutations, Y389C and V409I in their *narK* transporter genes, *narK1* and *narK4*, respectively (Table 3). A mutation (P18L) in the gene, *narL*, that possibly codes for nitrate/nitrite response transcriptional regulatory protein, NARL, was observed in all L6 isolates.

The nitrate concentrations were determined by converting nitrate to nitrite and the concentration was plotted according to the three lineages (Fig 6). The mean concentrations of nitrate
for L4, L5 and L6 isolates were determined as 4.69 μM (SD = 0.8), 4.93 μM (SD = 0.9) and 4.30 μM (SD = 0.5), respectively. Although L5 isolates had the highest mean concentrations of nitrate, followed by L4 and L6, the difference was not statistically significant (p = 0.27). Genomic analysis of the mycobacterial isolates revealed several mutations in genes associated with nitrate reduction. A lineage-specific mutation, P18L, in the \textit{narL} which codes for Nitrate/Nitrite response transcriptional regulatory protein, NARL, was observed among all L6 isolates. Additional \textit{Rv0890c} mutations, A355T & V647G were observed in L6.C1 isolates and Q286R was observed in all L5 isolates (Table 3). We also observed mutations, S435R and E967G, in another nitrate reductase gene, \textit{narG}, in L5 and L6.C1 isolates. Both L5 and L6 showed a common mutation, D77G, in \textit{narX}, which codes for another nitrate reductase, NARX.

### Table 2. Mutational analysis of genes that may be associated with growth rate.

| Isolate       | Gene name | Mutation Description                              |
|---------------|-----------|--------------------------------------------------|
| L6.C3, L5.C2 & L5.C1 | \textit{ftsE} | R16P, Y98H & V75I Putative cell division ATP-binding protein FTSE (septation component-transport ATP-binding protein ABC transporter) |
| L6.2 (one isolate:1283) | \textit{hmp} | T125I Possible Hemoglobin-related protein HMP. Possible ketosteroid-9-alpha-hydroxylase. |
| L5 only       | \textit{pknB} | V251L Transmembrane Serine/Threonine-protein kinase B PKNB (Protein Kinase B) (STPK B) |
| Maf only      | \textit{pstP} | R3L Phosphoserine/Threonine Phosphatase PSTP |
| Maf only      | \textit{pstP} | S455A Phosphoserine/Threonine Phosphatase PSTP |
| L5.3 (one isolate: 2196) | \textit{pstP} | S475R Phosphoserine/Threonine Phosphatase PSTP |
| All except H37Rv | \textit{rpfE} | R126Q Probable resuscitation-promoting factor RPFE |
| L5.C1 only    | \textit{rpfE} | A87V Probable resuscitation-promoting factor RPFE |
| L6 only       | \textit{suhB} | D43A Possible extragenic suppressor protein SUHB |
| L5 only       | \textit{suhB} | A196G Possible extragenic suppressor protein SUHB |
| L5 only       | \textit{whiB3} | E71stop Transcriptional regulatory protein WHIB-like WHIB3 |

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Urease activity test

Urease activity of the three lineages was compared by measuring the absorbance at 630 nm at days 1, 3 and 7 (Fig 7). Each time point represented an average of the representative mycobacterial isolates of the three different lineages (Table 1). This experiment included H37Rv which is known to be positive for urease test as well as the majority of the members of MTBC. The average urease activity of L4 (average urease activity = 0.190, SD = 0.02) and L6 (average urease activity = 0.192, SD = 0.02) isolates was stronger than L5 isolates (average urease activity = 0.187, SD = 0.02) isolates.

Fig 6. Comparison of nitrate concentration. The individual isolates are represented by the black points in the boxplot.

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activity = 0.167, SD = 0.01). We analyzed the difference in the urease activity at day 7 for the isolates. There was significant difference (p = 0.02) between the average urease activity of the positive control, H37Rv (average urease activity = 0.211, SD = 0.02) and the negative control (average urease activity = 0.161, SD = 0.008). The same trend was observed between H37Rv and L5 (p = 0.02). Two mutations, G98D and Y169C, in the Rv1395 gene which is associated with a transcriptional regulatory protein were observed in L5.C2 strains (Table 4).

**Tween hydrolysis test**

We determined the ability of the mycobacterial isolates to hydrolyze tween by measuring the optical density at 630 nm at days 1, 3, 5 and 7. *Mycobacterium aurum* represented by the purple line was positive whereas the negative control presented by the blue line which is the tween hydrolysis reagent only was negative (Fig 8). All 3 lineages were negative for the tween hydrolysis test. Since all the lineages were negative for tween hydrolysis, mutation analyses for genes that may be involved in Tween hydrolysis were excluded from this study.

**Thiophen-2-carboxylic acid hydrazide (TCH) susceptibility**

Susceptibility pattern of the mycobacterial isolates against low concentrations of TCH was determined with a clinical strain of *M. bovis* used as a control. All mycobacterial isolates used were resistant to TCH except one strain of L6 (Table 5). Genomic analysis revealed a mutation, A11D, in *furA* gene in L5.C2. There was no additional mutation found in the analysed genes of the isolates susceptible to TCH.

![Fig 7. Comparison of urease activity of the three different lineages. A negative control depicted by the blue line was only urea broth. H37Rv was included in the experimental set-up as a positive control (black line).](https://doi.org/10.1371/journal.pone.0255433.g007)

| Lineage | Gene  | Mutation          | Gene function                                      |
|---------|-------|-------------------|----------------------------------------------------|
| L5.C2   | Rv1395| G98D & Y169C      | Transcriptional Regulatory Protein                 |
| L5.C1   | mrsA  | T125I             | Probable Phospho-Sugar Mutase/Mrsa Protein Homolog  |

| Table 4. Genomic analysis for urease activity test. |
Mycobacterium africanum (Maf) is mainly restricted to West Africa for reasons that are not well understood. Previously, studies have delineated distinct phenotypic characteristics such as the association of Maf L5 with ethnicity whereas Maf L6 has been associated with HIV infection and slower progression to active disease [18,39,41,52]. Attempts have been made to further explore these observations with comparative genomics and molecular epidemiology with limited phenotypic studies. Some previous studies involving Maf L6 showed characteristics such as slow growth, preference for pyruvate as a carbon source in a liquid medium and dysgonic growth nature on solid medium [34,44,53,54]. This study therefore, sought to

![Fig 8. Tween hydrolysis test. A negative control depicted by the blue line had only the Tween hydrolysis reagent. *Mycobacterium aurum* represented by the purple line was included in the experimental set-up as a positive control.](https://doi.org/10.1371/journal.pone.0255433.g008)

**Discussion**

*Mycobacterium africanum* (Maf) is mainly restricted to West Africa for reasons that are not well understood. Previously, studies have delineated distinct phenotypic characteristics such as the association of Maf L5 with ethnicity whereas Maf L6 has been associated with HIV infection and slower progression to active disease [18,39,41,52]. Attempts have been made to further explore these observations with comparative genomics and molecular epidemiology with limited phenotypic studies. Some previous studies involving Maf L6 showed characteristics such as slow growth, preference for pyruvate as a carbon source in a liquid medium and dysgonic growth nature on solid medium [34,44,53,54]. This study therefore, sought to

| Isolate ID | Sub-lineage | Lineage | TCH results | Gene | Mutation | Isolate ID | Sub-lineage | Lineage | TCH results | Gene | Mutation |
|------------|-------------|---------|-------------|------|----------|------------|-------------|---------|-------------|------|----------|
| H37Rv      |             |         | R           | -    | -        | 1283       | L6.2        | L6      | R           | -    | -        |
| *M. bovis* |             |         | S           | N/A  | N/A      | 1957       | L6.2        | L6      | R           | -    | -        |
| 1036       | Cameroon    | L4      | R           | -    | -        | 1102       | L6.2        | L6      | R           | -    | -        |
| 1034       | Cameroon    | L4      | R           | -    | -        | 1434       | L6.3        | L6      | R           | -    | -        |
| 1327       | Cameroon    | L4      | R           | -    | -        | 2150       | L6.3        | L6      | S           | -    | -        |
| 1307       | Cameroon    | L4      | R           | -    | -        | 1289       | L6.3        | L6      | R           | -    | -        |
| 1346       | Cameroon    | L4      | R           | -    | -        | 1421       | L5.1        | L5      | R           | -    | -        |
| 1979       | Ghana       | L4      | R           | -    | -        | 2910       | L5.2        | L5      | R           | furA | A11D     |
| 2070       | Ghana       | L4      | R           | -    | -        | 1010       | L5.2        | L5      | R           | furA | A11D     |
| 1800       | Ghana       | L4      | R           | -    | -        | 1786       | L5.1        | L5      | R           | -    | -        |
| 1608       | Ghana       | L4      | R           | -    | -        | 2541       | L5.3        | L5      | R           | -    | -        |
| 1448       | Ghana       | L4      | R           | -    | -        | 2196       | L5.3        | L5      | R           | -    | -        |
| 1280       | L6.1        | L6      | R           | -    | -        | 2384       | L5.4        | L5      | R           | -    | -        |
| 1821       | L6.1        | L6      | R           | -    | -        | 1984       | L5.4        | L5      | R           | -    | -        |
| 2016       | L6.1        | L6      | R           | -    | -        | 1144       | L5.5        | L5      | R           | -    | -        |
| 1082       | L6.2        | L6      | R           | -    | -        | 1313       | L5.5        | L5      | R           | -    | -        |

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phenotypically characterize L4, L5 and L6 which are the three most dominant MTBC genotypes in West Africa [34,53]. We carried out mutation analyses of the genes involved in the phenotypic assays. However, this study did not carry out complementation experiments to confirm the effects of the observed mutations.

We confirmed the preference of Maf lineages for pyruvate as a carbon source compared to glycerol although L5 also grows fairly well with glycerol (Fig 1). This observation confirms previous comparative genomic studies that revealed the presence of a single nucleotide polymorphism (SNP), E220D, in the pykA gene of M. africanum and M. bovis which impairs the activity of pyruvate kinase in the metabolism of carbohydrates [44,47,55]. Another SNP, R179S, in the eno gene was found to be associated with only L6 which could contribute to the more impaired utilization of glycerol among L6 strains [55].

On the other hand, the observed reduced growth of L4 on pyruvate supplemented media was expected: confirming the loss of ATP for skipping the glycolytic pathway. The difference in growth rate of the 3 lineages on pyruvate was minimal compared to the high disparity observed on glycerol. Hence, for a comparative phenotypic study on L-J slants, it is recommended to use pyruvate as a carbon source to reduce biases. We again observed a higher growth rate for L4 compared to L5 and L6 on 7H11+G and 7H11only media.

The high growth rate of L4 in liquid medium observed in this study is consistent with other studies that reported a longer doubling time or slower growth for Maf from a defined inoculum [33,34,53]. This also confirms the observation made by Castete et al when they used biochemical assays to differentiate Maf from Mtb [56]. They also observed a longer time to positivity for Maf. We hypothesize that the slow growth of Maf could be due to mutations in some essential growth genes. In this study, mutations in fisE, pstP, whiB3 and suhB genes were detected among the Maf lineages. These genes are associated with cell division and are responsible for control of cell cycle and virulence in bacteria. Thus, amino acid mutations in these genes could lead to defects in cell wall and cell division [57,58]. A future complementation experiments will be needed to validate the effects of these mutations.

Nitric oxide (NO) is a key anti-mycobacterial molecule which plays an important role in the pathogenesis of Mtb [59]. The amount of NO elicited by infected macrophages significantly contributes to the outcome of infection: the less the concentration of NO, the more virulent the pathogen [60]. A study where human macrophages were infected with Mtb under oxygen tension surprisingly showed high concentrations of nitrite from the activity of Mtb instead of the macrophages themselves [61]. The MTBC strains responded to the high concentration of nitrite by stopping their growth. Based on this finding, we hypothesized that; L6 will produce the highest concentration of nitrite/nitrate since it has the lowest growth rate. Interestingly, the reverse was observed for L6 which could be due to a mutation, P18L, in narL which codes for nitrate/nitrite response transcriptional regulatory protein, NarL. (Table 3). This specific mutation in narL has been reported to be associated with L6 strains [62]. According to a KEGG pathway analysis of NarL protein family, impaired NarL expression leads to the impairment of NarGHIJ (nitrate reductase) and ultimately results in reduced nitrite/nitrate concentrations [63].

Nitrogen metabolism of the three lineages was further assessed using urease activity. MTBC urease activity is classified as a virulence factor due to its alkalisation of acidic environment to aid the survival of MTBC [64–66]. Although urease activity is frequently used to differentiate MTBC from other mycobacteria, urea can be used as an alternative source of nitrogen by the MTBC especially in the absence of other nitrogen sources but the levels of utilization may differ depending on the genotypes [67,68]. The highest urease activity was observed in L6 isolates, and this suggests L6 prefers to utilize the alternative pathway by converting urea to carbon dioxide and ammonia [69] instead of nitric oxide production. From comparative mutational
analysis of genes associated with urease activity, we identified 3 mutations affecting 2 genes (Table 4). However, the mutations were found among only L5 strains which may explain the limited urease activity of L5 compared to the other genotypes.

Lipids form an important component of the life cycle of MTBC and hence, lipases and esterases are crucial for its pathogenesis [70]. The MTBC lipases hydrolyze host cell lipids into fatty acids which they use as energy source and building material for replication [71,72]. Tween hydrolysis test was carried out to characterize the hydrolytic properties of lipases/esterases of the three different lineages. All three lineages were negative for the tween hydrolysis test as expected for MTBC strains. This observation suggests the use of Tween 80 in Mycobacterial cultures not exceeding 20 days will likely not interfere with MTBC metabolism but rather serve its primary purpose as a non-ionic surfactant [73,74].

Lastly, the ability of mycobacteria to grow in the presence of inhibitory substances such as TCH was used to determine the diversity in virulence. Only one L6 strain was susceptible to TCH which is quite interesting because L6 are phylogenetically more closely related to M. bovis than other human adapted MTBC lineages [39].

Conclusion

In conclusion, L4 grows faster than Maf (L5 & L6) and even between the two Maf lineages; L5 grows faster than L6. The other assays carried out in this study also confirm the phenotypic diversity in the three lineages. There was generally significant diversity in the genotypic and phenotypic profiles of the MTBC lineages and the observed phenotypic variations might be explained by mutations identified by the comparative genomic analyses carried out. However, complementation studies need to be carried out to appropriately correlate the observed mutations with the different phenotypic characteristics. This study confirms the earlier reported genomic variations among the lineages which may potentially have implications on microbial physiology. The diversity in pathogen physiology can be used to explain the clinical and epidemiological characteristics of Maf.

Supporting information

S1 Table. Accession numbers for the analysed genes.
(XLSX)

S2 Table. List of genes used for the study.
(XLSX)

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References
1. WHO. Global Tuberculosis report. 2020.
2. Velayati AA, Farnia P. Chapter 1—The Species Concept. In: Velayati AA, Farnia P, editors. Atlas of Mycobacterium Tuberculosis. Boston: Academic Press; 2017. p. 1–16.
3. Ngabonziza JCS, Loiseau C, Marceau M, Jouet A, Menardo F, Tzfadia O, et al. A sister lineage of the Mycobacterium tuberculosis complex discovered in the African Great Lakes region. Nature Communications. 2020; 11(1):1–11.
4. Gagneux S. Ecology and evolution of Mycobacterium tuberculosis. Nature Reviews Microbiology. 2018; 16(4):202. https://doi.org/10.1038/nrmicro.2018.8 PMID: 29456241
5. Gagneux S, Small PM. Global phylogeography of Mycobacterium tuberculosis and implications for tuberculosis product development. The Lancet Infectious diseases. 2007; 7(5):328–37. https://doi.org/10.1016/S1473-3099(07)70108-1 PMID: 17448936
6. Coscolla M, Gagneux S, Menardo F, Loiseau C, Ruiz-Rodriguez P, Borrell S, et al. Phylogenomics of Mycobacterium africanum reveals a new lineage and a complex evolutionary history. 2021; 7(2).
7. Bottai D, Frigui W, Sayes F, Di Luca M, Spadoni D, Pawlik A, et al. TbD1 deletion as a driver of the evolutionary success of modern epidemic Mycobacterium tuberculosis lineages. Nature Communications. 2020; 11(1):684. https://doi.org/10.1038/s41467-020-14508-5 PMID: 32019932
8. Brosch R, Gordon SV, Marmiesse M, Brodin P, Eiglmeier K, et al. A new evolutionary scenario for the Mycobacterium tuberculosis complex. 2002; 99(6):3684 –9.
9. Fransen F, Heckenberg SG, Hamstra HJ, Feller M, Boog CJ, van Putten JP, et al. Naturally occurring lipid A mutants in Neisseria meningitidis from patients with invasive meningococcal disease are associated with reduced coagulopathy. PLoS pathogens. 2009; 5(4):e1000396. https://doi.org/10.1371/journal.ppat.1000396 PMID: 19390612
10. van Loo IH, van der Heide HG, Nagelkerke NJ, Verhoef J, Mooi FR. Temporal trends in the population structure of Bordetella pertussis during 1949–1996 in a highly vaccinated population. The Journal of infectious diseases. 1999; 179(4):915 –23. PMID: 10068587
11. Schols L, Van der Heide H, Witteveen S, Zomer B, Van der Ende A, Burger M, et al. Two variants among Haemophilus influenzae serotype b strains with distinct bcs4, hcsA and hcsB genes display differences in expression of the polysaccharide capsule. BMC microbiology. 2008; 8(1):35. https://doi.org/10.1186/1471-2180-8-35 PMID: 18298818
12. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rümke H, et al. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. The Lancet. 2004; 363(9424):1871–2. https://doi.org/10.1016/S0140-6736(04)16357-5 PMID: 15183627
13. Coscolla M, Gagneux S. Does M. tuberculosis genomic diversity explain disease diversity? Drug discovery today Disease mechanisms. 2010; 7(1):e43–e69. https://doi.org/10.1016/j.ddmec.2010.09.004 PMID: 21076640
14. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. 2018; 31(4):e00088–17.
15. Gyles C, Boerlin P. Horizontally Transferred Genetic Elements and Their Role in Pathogenesis of Bacterial Disease. 2014; 51(2):328–40.

16. Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. Clinical & Experimental Immunology. 2003; 133(1):30–7. https://doi.org/10.1046/j.1365-2249.2003.02171.x PMID: 12823275

17. Dale JW. Mobile genetic elements in mycobacteria. The European respiratory journal Supplement. 1995; 20:633s–48s. PMID: 8590564

18. De Jong BC, Hill PC, Aiken A, Awine T, Martin A, Adetila IM, et al. Progression to active tuberculosis, but not transmission, varies by Mycobacterium tuberculosis lineage in The Gambia. The Journal of infectious diseases. 2008; 198(7):1037–43. https://doi.org/10.1086/591504 PMID: 18702608

19. Asare P, Asante-Poku A, Prahl DA, Borrell S, Osei-Wusu S, Otchere ID, et al. Reduced transmission of Mycobacterium africanum compared to Mycobacterium tuberculosis in urban West Africa. International Journal of Infectious Diseases. 2018. https://doi.org/10.1016/j.ijid.2018.05.014 PMID: 29879521

20. Tientcheu LD, Koch A, Ndengane M, Andoeseh G, Kampmann B, Wilkinson RJ. Immunological consequences of strain variation within the Mycobacterium tuberculosis complex. Eur J Immunol. 2017; 47 (3):432–45. https://doi.org/10.1002/eij.201646562 PMID: 28150302

21. Nguyen VAT, Bafuls A-L, Tran THT, Pham KLT, Nguyen TS, Nguyen HV, et al. Mycobacterium tuberculosis lineages and anti-tuberculosis drug resistance in reference hospitals across Viet Nam. BMC microbiology. 2016; 16(1):167-. https://doi.org/10.1186/s12866-016-0784-6 PMID: 27464737

22. Orgeur M, Frigui W, Pawlik A, Clark S, Williams A, Ates LS, et al. Pathogenomic analyses of Mycobacterium microti, an ESX-1-deleted member of the Mycobacterium tuberculosis complex causing disease in various hosts. 2021; 7(2). https://doi.org/10.1099/mgen.0.000505 PMID: 33529148

23. Victor TC, Streicher EM, Kewley C, Jordaan AM, van der Spuy GD, Bosman M, et al. Spread of an emerging Mycobacterium tuberculosis drug-resistant strain in the Western Cape of South Africa. The International Journal of Tuberculosis and Lung Disease. 2007; 11(2):195–201. PMID: 17263291

24. Forreillard MA, Klepp LI, Giffre A, Sabio y García J, Morbidoni HR, de la Paz Santangelo M, et al. Virulence factors of the Mycobacterium tuberculosis complex. Virulence. 2013; 4(1):3–66. https://doi.org/10.4161/viru.22329 PMID: 23076359

25. Zhao QJ, Xie JP. Mycobacterium tuberculosis proteases and implications for new antibiotics against tuberculosis. Critical reviews in eukaryotic gene expression. 2011; 21(4):347–61. https://doi.org/10.1615/critrevukargeneexpr.v21.i4.50 PMID: 22181704

26. Hanekom M, Gey van Pittius NC, McEvoy C, Victor TC, Van Helden PD, Warren RM. Mycobacterium tuberculosis Beijing genotype: A template for success. Tuberculosis. 2011; 91(6):510–23. https://doi.org/10.1016/j.tube.2011.07.005 PMID: 21835699

27. Niemann S, Diel R, Khechinashvili G, Gega M, Mdivani N, Tang YW. Mycobacterium tuberculosis Beijing lineage favors the spread of multidrug-resistant tuberculosis in the Republic of Georgia. J Clin Microbiol. 2010; 48(10):544–50. https://doi.org/10.1128/JCM.00715-10 PMID: 20702677

28. Bentley SD, Comas I, Bryant JM, Walker D, Smith NH, Harris SR, et al. The genome of Mycobacterium africanum West African 2 reveals a lineage-specific locus and genome erosion common to the M. tuberculosis complex. PLoS neglected tropical diseases. 2012; 6(2):e1552. https://doi.org/10.1371/journal.pntd.0001552 PMID: 22389744

29. Stamm CE, Pasko BL, Chaisavaneyakom S, Franco LH, Nair VR, Weigele BA, et al. Screening Mycobacterium tuberculosis Secreted Proteins Identifies Mpt64 as a Eukaryotic Membrane-Binding Bacterial Effector. mSphere. 2019; 4(3). https://doi.org/10.1128/mSphere.00354-19 PMID: 31167949

30. Dunphy KY, Senaratne RH, Masuzawa M, Kendall LV, Riley LW. Attenuation of Mycobacterium tuberculosis functionally disrupted in a fatty acyl-coenzyme A synthetase gene fadD5. The Journal of infectious diseases. 2010; 201(8):1232–9. https://doi.org/10.1086/651452 PMID: 20214478

31. Marrero J, Trujillo C, Rhee KY, Eht S. Glucose phosphorylation is required for Mycobacterium tuberculosis persistence in mice. PLoS pathogens. 2013; 9(1):e1003116–e. https://doi.org/10.1371/journal.ppat.1003116 PMID: 23326232

32. Mann FM, Xu M, Davenport EK, Peters RJ. Functional characterization and evolution of the isocitrosin operon in Mycobacterium tuberculosis and related Mycobacteria. Frontiers in microbiology. 2012; 3:368-. https://doi.org/10.3389/fmicb.2012.00368 PMID: 23091471

33. Bold TD, Davis DC, Penberthi KK, Cox LM, Ernst JD, de Jong BC. Impaired fitness of Mycobacterium africanum despite secretion of ESAT-6. Journal of Infectious Diseases. 2012; 205(6):984–90. https://doi.org/10.1093/infdis/jir883 PMID: 22301632

34. Ates LS, Dippenaar A, Sayes F, Pawlik A, Bouchier C, Ma L, et al. Unexpected genomic and phenotypic diversity of Mycobacterium africanum lineage 5 affects drug resistance, protein secretion,
35. de Jong BC, Hill PC, Brookes RH, Otu JK, Peterson KL, Small PM, et al. Mycobacterium africanum: a new opportunistic pathogen in HIV infection? Aids. 2005; 19(15):1714–5. https://doi.org/10.1097/01.aids.0000185991.54594.51 PMID: 16184053
36. Otchere ID, Coscolla M, Sánchez-Busó L, Asante-Poku A, Brites D, Loiseau C, et al. Comparative genomics of Mycobacterium africanum Lineage 5 and Lineage 6 from Ghana suggests distinct ecological niches. Scientific reports. 2018; 8(1):1–11.
37. Sanoussi CND, Coscolla M, Ofori-Anyinam B, Otchere ID, Antonio M, Niemann S, et al. Comparative genomics of Mycobacterium africanum Lineage 5 and Lineage 6 from Ghana suggests distinct ecological niches. Scientific reports. 2018; 8(1):1–11.
38. Yeboah-Manu D, Asare P, Asante-Poku A, Otchere ID, Osei-Wusu S, Danso E, et al. Spatio-Temporal Distribution of Mycobacterium tuberculosis Complex Strains in Ghana. PLoS One. 2016; 11(8):e0161892. https://doi.org/10.1371/journal.pone.0161892 PMID: 27564240
39. Yeboah-Manu D, Bodmer T, Mensah-Quainoo E, Owusu S, Ofori-Adjei D, Hattendorf J, et al. Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. Journal of Clinical Microbiology. 2004; 42(12):5875–6. https://doi.org/10.1128/JCM.42.12.5875-5876.2004 PMID: 15583329
40. Asante-Poku A, Yeboah-Manu D, Otchere ID, Aboagye SY, Stucki D, Hattendorf J, et al. Mycobacterium africanum is associated with patient ethnicity in Ghana. PLoS neglected tropical diseases. 2015; 9(1):e3370. https://doi.org/10.1371/journal.pntd.0003370 PMID: 25569290
41. Otchere ID, Asante-Poku A, Osei-Wusu S, Baddoo A, Sarpong E, Ganiyu AH, et al. Detection and characterization of drug-resistant conferring genes in Mycobacterium tuberculosis complex strains: A prospective study in two distant regions of Ghana. Tuberculosis (Edinb). 2016; 99:147–54. https://doi.org/10.1016/j.tube.2016.05.014 PMID: 27450017
42. Stinson K, Eisenach K, Kayes S, Matsumoto M, Siddiqi S, Nakashima S. Mycobacteriology Laboratory Manual, global laboratory initiative advancing TB diagnosis. April; 2014.
43. Keating LA, Wheeler PR, Mansoor H, Inwald JK, Dale J, Hewinson RG, et al. The pyruvate requirement of some members of the Mycobacterium tuberculosis complex is due to an inactive pyruvate kinase: implications for in vivo growth. Molecular microbiology. 2005; 56(1):163–74. https://doi.org/10.1111/j.1365-2958.2005.04524.x PMID: 15773987
44. Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med. 2007; 43(5):645–57. https://doi.org/10.1016/j.freeradbiomed.2007.04.026 PMID: 17664129
45. Lew JM, Kapopoulou A, Jones LM, Cole ST. Tuberculist–10 years after. Tuberculosis. 2011; 91(1):1–7. https://doi.org/10.1016/j.tube.2010.09.008 PMID: 20980199
46. Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998; 393(6685):537–44. https://doi.org/10.1038/31159 PMID: 9634230
47. Rice P, Longden I, Bleasby A. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. 2000; 16(6):276–7. https://doi.org/10.1016/s0168-9525(00)02024-2 PMID: 10827456
48. Wickham H, Ggplot2: Elegant graphics for data analysis. Springer International Publishing. 2016(Use R!).
49. Wickham H, Hester J, R F. readr: Read Rectangular Text Data. 2017.
50. Wickham H, Hester J, R F. readr: Read Rectangular Text Data. 2017.
51. Wickham H. The Split-Apply-Combine Strategy for Data Analysis. Journal of Statistical Software. 2011; 40:1–29.
52. Asante-Poku A, Otchere ID, Osei-Wusu S, Sarpong E, Baddoo A, Forson A, et al. Molecular epidemiology of Mycobacterium africanum in Ghana. BMC infectious diseases. 2016; 16(1):385. https://doi.org/10.1186/s12879-016-2756-6 PMID: 2756391
53. Gehre F, Otu J, DeRiemer K, de Sessions PF, Hibberd ML, Mulders W, et al. Deciphering the growth behaviour of Mycobacterium africanum. PLoS neglected tropical diseases. 2013; 7(5):e2220. https://doi.org/10.1371/journal.pntd.0002220 PMID: 23696111
54. de Jong BC, Adetifa I, Walther B, Hill PC, Antonio M, Ota M, et al. Differences between tuberculosis cases infected with Mycobacterium africanum, West African type 2, relative to Euro-American Mycobacterium tuberculosis: an update. FEMS immunology and medical microbiology. 2010; 58(1):102–5. https://doi.org/10.1111/j.1574-695X.2009.00628.x PMID: 2002176
55. Ofori-Anyinam B, Riley AJ, Jobarteh T, Gitteh E, Sarr B, Faal-Jawara TI, et al. Comparative genomics shows differences in the electron transport and carbon metabolic pathways of Mycobacterium africanaum relative to Mycobacterium tuberculosis and suggests an adaptation to low oxygen tension. Tuberculosis. 2020; 120:101899. https://doi.org/10.1016/j.tube.2020.101899 PMID: 32090860

56. Castets M, Boisvert H, Grumbach F, Brunel M, Rist N. Tuberculosis bacilli of the African type: preliminary note. Revue de tuberculose et de pneumologie. 1968; 32(2):179–84. PMID: 4985104

57. Sharma AK, Arora D, Singh LK, Gangwal A, Saigal A, Mollie V, et al. Serine/threonine protein phosphatase PstP of Mycobacterium tuberculosis is necessary for accurate cell division and survival of pathogen. Journal of Biological Chemistry. 2016; 291(46):24215–30. https://doi.org/10.1074/jbc.M116.754531 PMID: 27758870

58. Iswahyudi, Mukamolova GV, Straatman-Iwanowska AA, Alcock N, Ajuh P, Turapov O, et al. Mycobacterial phosphatase PstP regulates global serine threonine phosphorylation and cell division. Scientific Reports. 2019; 9(1):8337. https://doi.org/10.1038/s41598-019-44841-9 PMID: 31171861

59. Wayne L, Hayes L. Nitrate reduction as a marker for hypoxic shutdown of Mycobacterium tuberculosis. Tubercl and Lung Disease. 1998; 79(2):127–32. https://doi.org/10.1054/tuld.1998.0015 PMID: 10645451

60. Jamaati H, Mortaz E, Pajouhi Z, Folkerts G, Movassagh M, Moloudizargari M, et al. Nitric Oxide in the Pathogenesis and Treatment of Tuberculosis. Frontiers in microbiology. 2017; 8:2008-. https://doi.org/10.3389/fmicb.2017.02008 PMID: 29085351

61. Cunningham-Bussel A, Zhang T, Nathan CF. Nitrite produced by Mycobacterium tuberculosis in human macrophages in physiologic oxygen impacts bacterial ATP consumption and gene expression. Proceedings of the National Academy of Sciences. 2013; 110(45):E4256–E465. https://doi.org/10.1073/pnas.1316894110 PMID: 24145454

62. Rose GD. A genomic and transcriptomic study of lineage-specific variation in Mycobacterium tuberculosis: UCL (University College London); 2013.

63. Kanehisa M. Toward understanding the origin and evolution of cellular organisms. Protein science: a publication of the Protein Society. 2019; 28(11):1947–51. https://doi.org/10.1002/pro.3715 PMID: 31441146

64. Clemens DL, Lee BY, Horwitz MA. Puriﬁcation, characterization, and genetic analysis of Mycobacterium tuberculosis urease, a potentially critical determinant of host-pathogen interaction. J Bacteriol. 1995; 177(19):5644–52. https://doi.org/10.1128/jb.177.19.5644-5652.1995 PMID: 7559354

65. Mukai T, Maeda Y, Tamura T, Miyamoto Y, Makino M. CD4+ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant Mycobacterium bovis bacillus Calmette-Guérin. FEMS immunology and medical microbiology. 2008; 53(1):96–106. PMID: 18400013

66. Sendide K, Deghmene AE, Reyrat JM, Talal A, Hmama Z. Mycobacterium bovis BCG urease attenuates major histocompatibility complex class II trafﬁcking to the macrophage cell surface. Infection and immunity. 2004; 72(7):4200–9. https://doi.org/10.1128/IAI.72.7.4200-4209.2004 PMID: 15213164

67. Steadham JE. Reliable urease test for identiﬁcation of mycobacteria. Journal of clinical microbiology. 1979; 10(2):134–7. https://doi.org/10.1128/jcm.10.2.134-137.1979 PMID: 389943

68. Lin W, Mathys V, Ang ELY, Koh VHQ, Martinez Gómez JM, Ang MLT, et al. Urease activity represents an alternative pathway for Mycobacterium tuberculosis nitrogen metabolism. Infection and immunity. 2012; 80(8):2771–9. https://doi.org/10.1128/iai.06195-11 PMID: 22645285

69. Habel JE, Bursey EH, Rho B-S, Kim C-Y, Segelke BW, Rupp B, et al. Structure of Rv1848 (UreA), the Mycobacterium tuberculosis urease subunit. Acta Crystallographica Section F: Structural Biology and Crystalization Communications. 2010; 66(7):781–6.

70. Rodríguez JG, Hernández AC, Helguera-Repetto C, Ayala DA, Guadarrama-Medina R, Anzóla JM, et al. Global adaptation to a lipid environment triggers the dormancy-related phenotype of Mycobacterium tuberculosis. MBio. 2014; 5(3). https://doi.org/10.1128/mBio.01255-14 PMID: 24846361

71. Vromman F, Subtil A. Exploitation of host lipids by bacteria. Current opinion in microbiology. 2014; 17:38–45. https://doi.org/10.1016/j.mib.2013.11.003 PMID: 24581691

72. Ehrn T, Schnappier D. Mycobacterium tuberculosis virulence: lipids inside and out. Nature medicine. 2007; 13(3):284–5. https://doi.org/10.1038/nm0307-284 PMID: 17342139

73. Meyers PR, Bourn WR, Steyn LM, van Helden PD, Beyers AD, Brown GD. Novel method for rapid measurement of growth of mycobacteria in detergent-free media. Journal of clinical microbiology. 1998; 36(9):2752–4. https://doi.org/10.1128/JCM.36.9.2752-2754.1998 PMID: 9705430

74. Stinson MW, Solotorovsky MJARoRD. Interaction of Tween 80 Detergent with Mycobacteria in Synthetic Medium: I. Effect of Tween 80 on the Growth and Turbidity Response of Mycobacterium avium Cultures. 1971; 104(5):717–27.