Tuberculosis 104 (2017) 58–64

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

Significant under expression of the DosR regulon in *M. tuberculosis* complex lineage 6 in sputum

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ARTICLE INFO

Article history:
Received 18 November 2016
Received in revised form
21 February 2017
Accepted 2 March 2017

Keywords:
Mycobacterium africanum
Mycobacterium tuberculosis
Hypoxia
Sputum
Gene expression
DosR

ABSTRACT

Mycobacterium africanum lineage (L) 6 is an important pathogen in West Africa, causing up to 40% of pulmonary tuberculosis (TB). The biology underlying the clinical differences between *M. africanum* and *M. tuberculosis* sensu stricto remains poorly understood. We performed *ex vivo* expression of 2179 genes of the most geographically dispersed cause of human TB, *M. tuberculosis* L4 and the geographically restricted, *M. africanum* L6 directly from sputa of 11 HIV-negative TB patients from The Gambia who had not started treatment. The DosR regulon was the most significantly decreased category in L6 relative to L4. Further, we identified nonsynonymous mutations in major DosR regulon genes of 44 L6 genomes of TB patients from The Gambia and Ghana. Using Lebek's test, we assessed differences in oxygen requirements for growth. L4 grew only at the aerobic surface while L6 grew throughout the medium. In the host, the DosR regulon is critical for *M. tuberculosis* in adaptation to oxygen limitation. However, *M. africanum* L6 appears to have adapted to growth under hypoxic conditions or to different biological niches. The observed under expression of DosR in L6 fits with the genomic changes in DosR genes, microaerobic growth and the association with extrapulmonary disease.

1. Introduction

Mycobacterium africanum lineages (L) 5 and 6 and *M. tuberculosis* sensu stricto (L1-L4, L7) belong to the *M. tuberculosis* complex (MTBC) and co-evolved with distinct human populations [1–4]. Whereas the global spread of *M. tuberculosis* lineages 1–4 was associated with urbanization and population expansion, the

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http://dx.doi.org/10.1016/j.tube.2017.03.001

two lineages of *M. africanum*, for reasons still unknown, primarily remain geographically restricted to West Africa. Interestingly, *M. tuberculosis* is relatively more virulent than *M. africanum* L6 as evidenced by significantly faster progression, in contacts of infectious cases, to active tuberculosis (TB) disease [5]. *M. africanum* L6 more commonly causes disease in persons with HIV infection, older age and malnutrition, implying a more opportunistic pathogen [6–8]. Furthermore, *M. africanum* lineages grow markedly slower than *M. tuberculosis* [9–10]. Taken together, clear phenotypic contrasts exist between the lineages of *M. africanum* and *M. tuberculosis,* yet the biology underlying the observed differences is still poorly understood.

Lacking an environmental reservoir, the proliferation of the MTBC relies on successful transmission from a diseased to a susceptible host via inhalation of aerosols containing bacilli. The bacteria maintain a complex life cycle enhancing transmission. Upon inhalation, bacilli reaching the lungs are engulfed by alveolar macrophages. Subsequently, infected macrophages induce immune responses leading to the recruitment of further immune cells and the formation of a granuloma. If the host is able to control the pathogen, the bacilli persist within the host for prolonged periods without causing disease. At this noninfectious stage, central metabolism and replication are shut down with bacilli moving into a state of dormancy until conditions for active replication, such as immunosuppression, return [11]. During active TB, when the host is contagious, granuloma maturation occurs leading to the release of bacilli into airways and subsequent expectoration as infectious aerosols [11]. To overcome this infection-to-transmission bottleneck, the bacterium adapted to all stages in its life cycle and developed numerous strategies, including the ability to rapidly adjust to changes from extracellular aerobic environments to hypoxic and nutrient-limited conditions commonly encountered during intracellular survival.

Mycobacterium tuberculosis, although classified as an obligate aerobe, survives during anaerobiosis. In the Wayne model, where cultures undergo gradual self-generated oxygen depletion and shift through microaerobic to anaerobic conditions, bacilli stop growing but survive [12]. The well-studied *M. tuberculosis* DosR regulon is crucial in this regard, as it controls the adaptation to oxygen limitation and has also been linked to virulence [13–14]. A recent study described roles for a number of metabolic pathways in the reference strain H37Rv (*M. tuberculosis* L4) by demonstrating regulatory networks were built around DosR (DevR) and Rv0081, indicating they are the major sensors orchestrating the switch from aerobic to anaerobic survival [15]. Further, *dosR* (*devR*) was upregulated during hypoxia and downregulated upon reaeration, a switch that can occur within 5 min [14–15]. *In vitro*, *dosR* is overexpressed in Beijing strains (lineage 2) [16–17]. However, little is known about the state and expression of genes within the DosR regulon of *M. africanum* and its response to hypoxia, although early literature indicated a preference for microaerobic growth [18].

Previous studies suggest a different intracellular survival strategy of *M. africanum* L6 when compared to *M. tuberculosis* evidenced by non-synonymous mutations in 5 of 7 operons essential for intracellular survival within host macrophages [10]. We here present results from an exploratory analysis in which we compared the *ex vivo* expression of 2179 genes of *M. tuberculosis* L4 and *M. africanum* L6 directly in sputa of HIV-negative TB patients. The DosR regulon was the most significantly differentially expressed category, with lower expression in *M. africanum* L6 relative to *M. tuberculosis* L4. Moreover, in a phenotypic analysis using Lebek's test for oxygen preference, we confirmed that *M. africanum* L6 grew microaerobically while *M. tuberculosis* L4 grew best aerobically. We also identified sequence polymorphisms in the differentially expressed genes, as well as related genes, in 44 *M. africanum* L6 genomes. Our results suggest that *M. africanum* L6 is less dependent on the DosR regulon and more adapted to a microaerobic lifestyle.

2. Materials and methods

2.1. Patients

Adults with new sputum smear positive TB in The Gambia were recruited between 2006 and 2009 and isolates were genotyped as previously described [19]. The study was approved by ethical committees in the Gambia, Stanford, and New York University and all patients provided written informed consent. For this analysis on strain differences, gene expression analysis on *M. tuberculosis* L4 and *M africanum* L6 from sputum was only performed if patients had not yet initiated treatment and were HIV negative. Eleven sputa from 5 *M. africanum* L6 and 6 *M. tuberculosis* L4 infected individuals were consecutively selected for analysis from a total of 27 patients with RNA of sufficient quality and quantity. The HIV status of patients infected with *M. africanum* L6 could only be confirmed for 3 of the 5 patients. Therefore, although gene expression results were available for the two patients with unconfirmed HIV status (Supplemental Table S1, Supplementary Material online), we excluded these from further analysis.

2.2. Sputum collection and gene expression

Spontaneously expectorated sputum was collected in guanidine thiocyanate (GTC) and resuspended in Trizol for RNA isolation and extraction using previously described methods [20]. We assayed expression of 2179 selected *M. tuberculosis* genes (54% of the genome) via multiplex quantitative RT-PCR (TaqMan) with a LightCycler 480 (Roche, Indianapolis, Indiana). Genotyping was performed on parallel sputum cultures as described previously [19].

Gene expression data was normalized using a median approach [15], a method appropriate for unpaired data with low levels of non-detection [21]. An unpaired, equal variance *t*-test was used to identify differential expression between *M. africanum* L6 and *M. tuberculosis* L4 strains. A modified Fisher's Exact test [22–23] was then performed on differentially expressed genes (p-value < 0.05) on TB specific categories [20] with Bonferroni multiple testing correction. Predicted gene functional annotations used in the supplemental tables were derived from MycoBASE and Tuberculist [22,24].

2.3. Detection of Single Nucleotide Polymorphisms

Single Nucleotide Polymorphisms (SNPs) within and between genes have the potential to affect gene expression. To ascertain if there were lineage specific mutations within genes of *M. africanum* L6, and whether the primers designed for L4 could bind L6, we compared SNPs in 44 *M. africanum* L6 strains isolated from TB patients both from The Gambia and Ghana with a reference dataset of SNPs in other lineages (Coscolla et al. in preparation). We used Burrows-Wheeler Aligner (BWA) to map Illumina reads against the MTBC reference genome described in Ref. [4]. BWA outputs were analyzed with SAMtools [25–26] to detect variable positions with respect to the reference genome. We applied heuristic filters to remove problematic positions. Filtering criteria were: Phred-scaled probability scores <20 and with read depth more than double the average read depth of the genome. Ambiguous base calls (i.e. more than one nucleotide called) were excluded. SNP lists for individual strains were combined into a single non-redundant database, and were annotated with ANNOVAR [27] using H37Rv annotation as a reference. SNPs in repeat-containing genes (REP13E12), family protein PE/PPE/PGRS, integrase, transposase resolvase, maturase, or

phage were excluded, and the final high-confidence list of SNPs was used to recover the corresponding base call for each genome.

2.4. Culture of M. africanum and M. tuberculosis in Lebek's medium

To determine if oxygen requirements for growth differed between *M. tuberculosis* L4 and *M. africanum* L6, Lebek's test for oxygen preference was carried out as described previously [18], except that the test was conducted in polypropylene-rather than glass tubes to comply with biosafety requirements. Briefly, a 2 mg/ml bacterial suspension was mixed with liquid agar based medium before it solidified, followed by incubation at 37 °C.

3. Results

3.1. DosR regulon gene expression in M. africanum L6

From all 2179 mycobacterial genes tested on sputa, the DosR regulon genes were the most differentially regulated category expressed between *M. africanum* L6 and *M. tuberculosis* L4 before and after multiple testing correction (Fig. 1, Supplemental Tables S1 and S2, Supplementary Material online). During the course of infection, *M. tuberculosis* encounters oxygen limitation. Under such conditions, the bacteria respond by switching on DosR, which is crucial for hypoxic response regulation in *M. tuberculosis*. As indicated by higher cycle threshold values, DosR regulated genes had lower expression in *M. africanum* L6 when compared to *M. tuberculosis* L4 (Fig. 2). On average, *M. tuberculosis* L4 had 2.5-fold higher expression of DosR regulon genes than *M. africanum* L6. The under expression of half (n = 26) of these genes was on average approximately 4 fold lower in *M. africanum* L6 and statistically significant (Supplemental Tables S2 and S3, Supplementary Material online). These included the main regulators encoded within the regulon, Rv0081 and DosR itself, as well as several conserved hypotheticals, implying a relatively lower requirement of these genes by *M. africanum* L6. Additionally, expression of the DosR-regulated nitrate transporter, *narK2* was significantly lower.

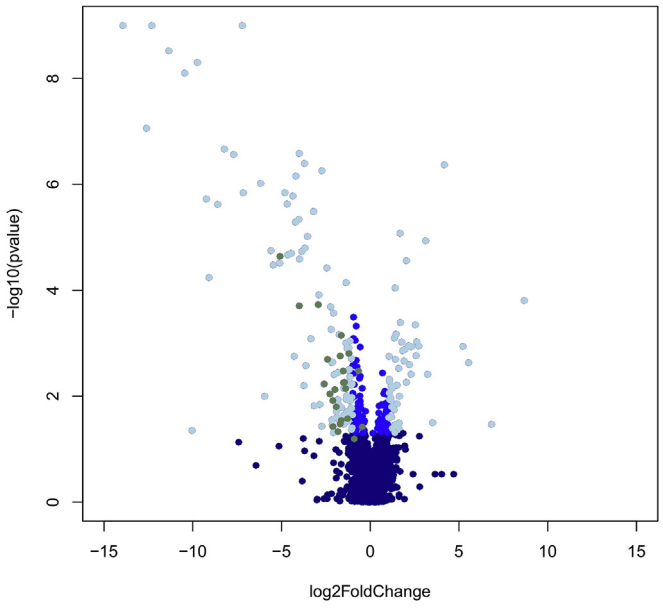

Fig. 1. Volcano plot of all genes tested visualizing fold change in expression and statistical significance. Dark blue dots indicate genes with no significant difference in expression between *M. africanum* L6 and *M. tuberculosis* L4. Mid-dark blue shows genes with unadjusted p value less than 0.05 and log 2 Fold change between −1 and 1. Light blue describes genes with unadjusted p value less than 0.05 and log 2 Fold change greater than (−)1. DosR genes significantly under expressed in *M. africanum* L6 relative to *M. tuberculosis* L4 following Bonferroni correction are shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

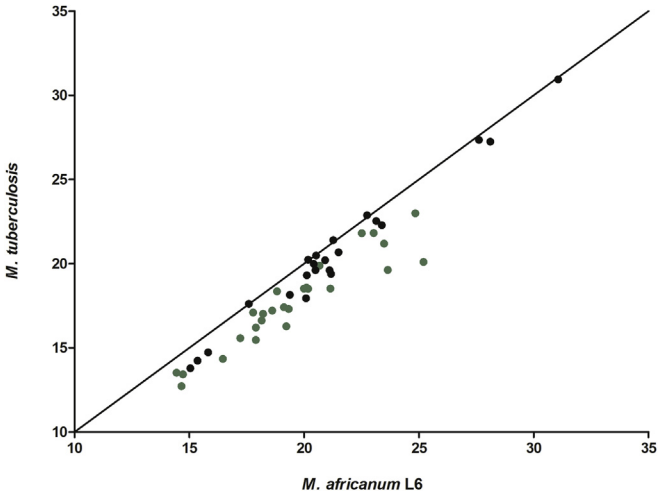

Fig. 2. Median qPCR cycle threshold values of the 48 dormancy regulon genes of *M. africanum* L6 and *M. tuberculosis* L4. Genes with significantly lower expression in *M. africanum* L6 relative to *M. tuberculosis* L4 are shown in green (same genes as the green ones in Fig. 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Detection of single nucleotide polymorphisms in major hypoxia response genes in M.africanum L6

Nucleotide polymorphisms within genes can affect gene function and potentially influence gene regulation when found in intergenic regions. The under expression of a significant number of DosR regulon genes in *M. africanum* L6 from sputa led us to assess the whole genome sequences of a collection of 44 *M. africanum* L6 strains for lineage-specific mutations within these genes. Notably, in all 44 strains, specific nonsynonymous SNPs were detected in *Rv0080* including the intergenic region of *Rv0080* and the gene encoding the regulatory hub in *M. tuberculosis* during hypoxia, *Rv0081* (Fig. 3). Although not under expressed in *M. africanum* L6 relative to *M. tuberculosis* L4, lineage-specific nonsynonymous SNPs were found in the *dosT* gene of all *M. africanum* L6 and also in *phoP/R* as described previously [28].

3.3. M. africanum L6 preferentially grew microaerobically while M. tuberculosis L4 grew aerobically

In Lebek's medium, classically used to assess oxygen preference between strains, we compared the growth of a clinical *M. tuberculosis* L4 strain, two *M. africanum* L6 clinical strains, and the *M. tuberculosis* reference strain H37Rv (L4). Both *M. africanum* L6 strains showed anaerobic growth below the surface while the clinical L4 strain and H37Rv showed growth only at the aerobic surface (Fig. 4). The results shown were confirmed in a technical replicate.

4. Discussion

Evidence points to important differences between the *in vitro* physiologic state of *M. tuberculosis* and the state of the bacteria in the human host [20,29–30]. Sputum is a valuable source to investigate the physiologic state of bacteria in the lung during

disease. A recent study indicates that *M. tuberculosis* transcription in sputum mirrors *M. tuberculosis* transcription in the lungs [30]. In the present study, we identified 2.5-fold lower expression of all DosR regulon genes in *M. africanum* L6 relative to *M. tuberculosis* L4 from sputa of HIV negative patients with TB disease. Key genes activated by *M. tuberculosis* in response to hypoxia, *dos R-S* and regulatory hub gene *Rv0081*, were significantly less expressed in *M. africanum* L6. Moreover, in all L6 strains sequenced we detected lineage-specific mutations in *Rv0080* and the intergenic region (possibly within the upstream promoter region) between *Rv0080* and *Rv0081*. The importance of *Rv0080* and the *Rv0080-Rv0081* intergenic region was shown in a previous study [31] in which DosR demonstrated binding to the intergenic region between *Rv0080* and *Rv0081*. Further, Rv0081 was found to bind an inverted repeat element located in its own upstream region. Our findings are significant and serve as a prelude to future studies because it is well

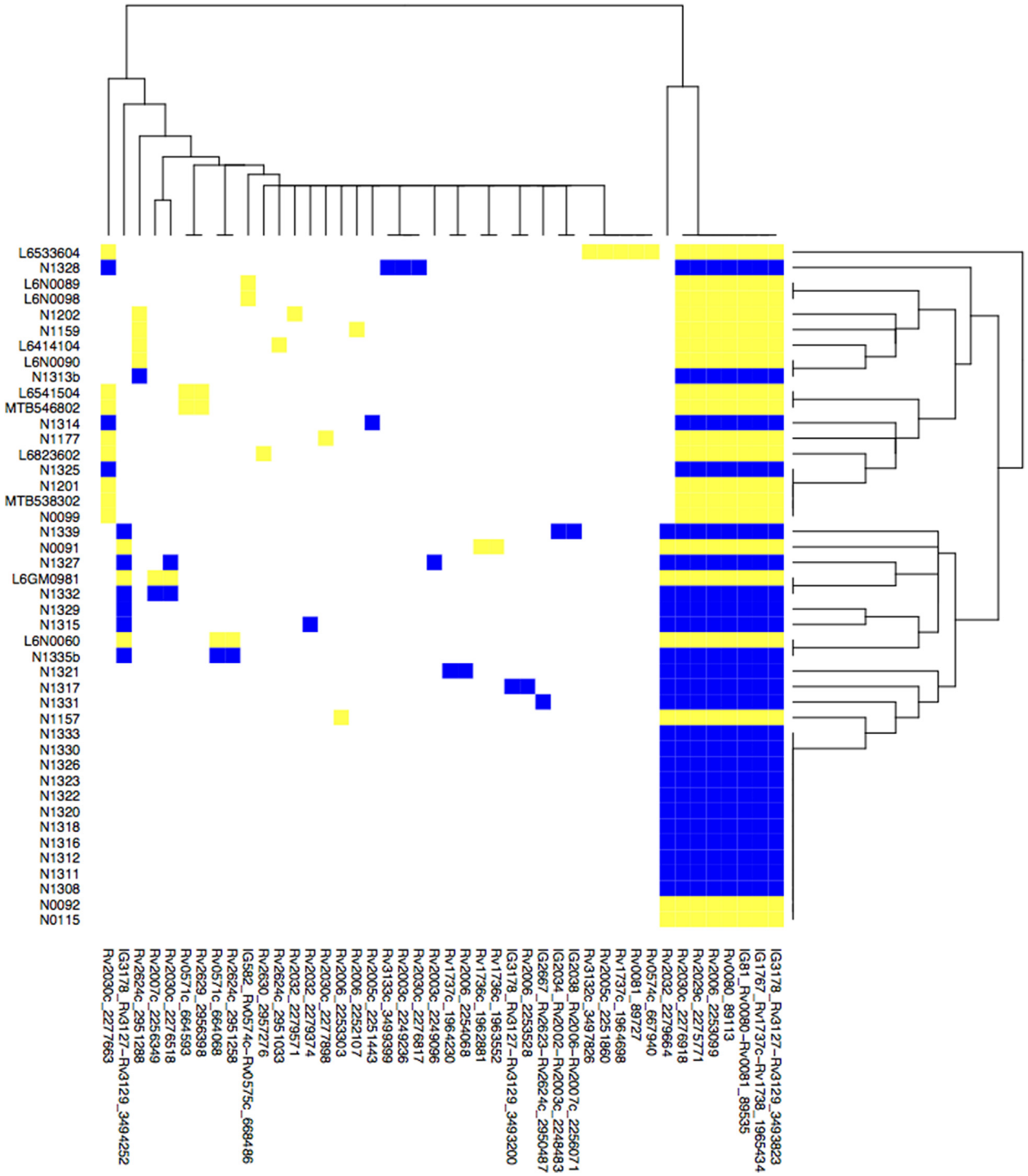

Fig. 3. Single Nucleotide Polymorphisms detected in 44 *M. africanum* L6 strains from Gambia and Ghana relative to H37Rv in under expressed genes. Blue and yellow indicate a SNP in Gambian and Ghanaian isolates respectively and white indicates wildtype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

known how *M. tuberculosis* uses the DosR regulon to quickly adjust to hypoxic stress, commonly encountered during intracellular survival within the macrophage and granuloma [11–12,32]. However, the state of DosR in *M. africanum* lineages is underexplored. Adaptation to all stages of a complex life cycle and versatility in the ability to switch between the different metabolic states through regulation of DosR could be a basis for higher pathogenicity of *M. tuberculosis*. Indeed, the constitutive over expression of DosR in *M. tuberculosis* W/Beijing (L2) is thought to contribute to the high virulence and transmissibility associated with this lineage [17].

As the DosR regulon has been linked to virulence, the observed under expression might contribute to the previously described differences between *M. africanum* L6 and *M. tuberculosis* lineages in clinical phenotype and disease progression [5]. Different animal models have been used to study TB infection and pathogenesis. In guinea pigs and rabbits, where hypoxic lesions develop [33], and in mice, *dosR-S* was required for full virulence. *dosR-S* mutants showed a growth defect and slower replication marked by lower counts of colony forming units in both lungs and spleen [34]. In a recent study in macaques, the closest experimental model to humans, Mehra and colleagues also reported the loss of clinical signs of TB, fever, progressive weight loss and radiographic lesions, in animals infected with *dosR* mutants [13]. Despite being TST positive, macaques infected with *dosR* mutants failed to develop clinical disease within the study period while those infected with wild type and complemented strains developed early TB. A statistically significant difference in survival between the wild type/complemented infected group and the group infected with mutants was reported. These observations confirm a major role for the full activity of DosR in conferring virulence within the host. Moreover, they bear striking similarity with the *M. africanum* L6 clinical phenotype which, when compared to *M. tuberculosis*, is attenuated, favors immunocompromised hosts, multiplies at a slower rate and is associated with slower progression to active disease [5,8,10]. The relative under expression of the DosR regulon in *M. africanum* L6 might thus be one possible explanation for the attenuated *M. africanum* L6 clinical phenotype.

The DosR regulon is controlled by the response regulator DosR and sensor kinases DosS (DevS) and DosT. The activation of DosR depends on both DosS and DosT. Honaker and colleagues showed the individual and collective importance of both sensors [35]. DosT, the first of the two sensors to respond to hypoxia and to activate DosR, is sensitive to oxygen and is a hypoxia sensor while DosS plays a role as a redox sensor. Therefore, it was suggested that DosT could be more important than DosS during early phase hypoxia particularly during the shift from an aerobic to hypoxic environment [36]. The response of DosT is short lived and quickly diminishes following the drop in oxygen levels. At this point DosS, also a member of the 48-gene DosR regulon, takes over and keeps DosR induction sustained and further induced [35–36]. In DosS and DosT single mutants, induction of the DosR regulon reduced by 45% showing that both DosT and DosS are essential for full induction of the DosR regulon during oxygen limitation [36]. In addition to the significant under expression of *dosS*, the mutated *dosT* gene in all *M. africanum* L6 strains analyzed could together contribute to the overall under expression of the DosR regulon in *M. africanum* L6.

dosR is regulated by *phoP* [15,37]. Mutations in the PhoPR system reportedly deactivate or interfere with proper functioning of the system and contribute to loss of virulence [38]. When the PhoR mutation common to *M. bovis* and *M. africanum* L6 was introduced in H37Rv, the Δ*phoPR::phoPR-bovis* mutant was marked by reduced bacillary load in human primary macrophages and severely impaired replication in immunocompetent BALB/c mice, which were reversed by complementation of the intact PhoPR gene. In the same study, the RD8 deletion, shared by *M. bovis* and *M. africanum* L6, was shown to 'rescue' the ESX-1 secretion system, which had been abrogated in the H37Rv Δ*phoPR* mutant [28]. In the present study, we confirmed nonsynonymous mutations in *phoP/R*. Mutations in *phoR* were detected in all Lineage 6 strains. Although no differential expression of *phoP*, *phoR*, or genes of the ESX-1 system was observed between *M. tuberculosis* L4 and *M. africanum* L6, based on the evidence that the *M. bovis* and *M. africanum* L6 *PhoR* mutation abrogated ESX-1 signaling [28], the reduced expression of the DosR regulon in *M. africanum* L6 is likely another downstream effect of the mutated PhoPR system.

Besides the clinical consequences, DosR regulon expression differences could reflect the respective bacterial physiologies and lifestyles. Increased DosR activity in *M. tuberculosis* might indicate greater requirement for genes associated with the switch to hypoxic survival. In contrast, it appears that *M. africanum* is naturally more capable of growing in hypoxic environments and does not rely on the regulatory switch to the same extent as *M. tuberculosis*. This assumption was further confirmed by our growth experiments, in which *M. africanum* L6 was able to grow aerobically and anaerobically throughout the whole Lebek medium (a test for oxygen preference), whereas *M. tuberculosis* L4 only grew on the

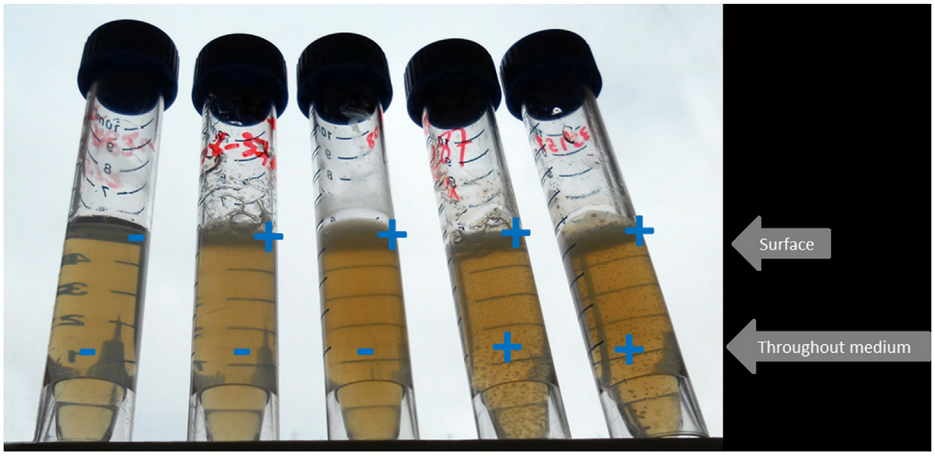

Fig. 4. Lebek test for oxygen preference. Test for oxygen preference in Lebek medium for the reference strain H37Rv (L4), a clinical L4 strain and 2 clinical *M. africanum* strains (L6). From left to right, tube 1, Negative control; tube 2, *M. tuberculosis* H37Rv; tube 3, L4 *M. tuberculosis* clinical strain (991508); tube 4, L6 *M. africanum* clinical strain (112287); tube 5, L6 *M. africanum* clinical strain (133158), showing diffused growth by *M. africanum* L6 compared to surface growth by *M. tuberculosis*.

surface of the slope, supporting the traditional classification of *M. africanum* L6 as microaerophilic [9,39–41]. The preference for microaerobic growth has been further supported in a study from 1973 in which paraffin embedded culture medium was used to show cross-sections of *M. africanum* colonies, which, unlike *M. tuberculosis* colonies that remain strictly at the surface, grew in the depth of the medium, explaining the umbilicated colony morphology [42]. The relative reduced responsiveness of the DosR regulon together with the observed preference for microaerobic growth of *M. africanum* L6 could either imply a preference for intracellular growth or adaptation to a fundamentally different biological niche within the host.

For instance, another key enzyme regulated by DosR during anaerobiosis in *M. tuberculosis* is a nitrate transporter encoded by *narK2* [43–45]. We also found that *narK2* mRNA is less abundant in *M. africanum* L6 when compared to *M. tuberculosis* L4. Traditional biochemical, microbiological assays are in line with our findings and showed that only a minority of *M. africanum* strains isolated from Ghana and Dakar were positive for intracellular nitrate, indicative of a general lack of NarK2 activity in *M. africanum* [18]. Our findings support both older and more recent MTBc speciation data describing *M. africanum* L6 as nitrate reductase negative [9,46].

Limitations of the present study include the fact that gene expression is averaged across all bacterial populations in sputum, and that the relatively small sample size and correction for multiple testing only allowed to detect sizeable differences in global expression levels. However, the detected differences between *M. africanum* L6 and *M. tuberculosis* L4 were still very significant, supporting the magnitude of the difference in expression of the DosR regulon between *M. tuberculosis* L4 and *M. africanum* L6. We detected additional differentially expressed genes in *M. africanum* L6 relative to *M. tuberculosis* L4 that did not reach statistical significance, possibly as a result of correcting for multiple testing using the more conservative Bonferroni method (Supplemental Table S2, Supplementary Material online). Sample quantities also did not permit mRNA analyses of host genes.

Although we show that *M. africanum* L6 is more capable of growth under hypoxic conditions reflected by microaerobic growth in Lebek's medium and the under expression of DosR regulon genes relative to *M. tuberculosis* L4, future studies need to demonstrate whether both pathogens would show these same differences in expression under identical hypoxic conditions. In a recent study where H37Rv (MTBC Lineage 4) was grown *in vitro* under hypoxic conditions in the Wayne model and subsequently subjected to gene expression analysis, dormancy regulon genes including *dosR* and *narK2* were overexpressed during non-replicating persistence-1 (NRP-1). Interestingly, *narK2* remained highly expressed even through NRP-2, emphasizing the importance of the dormancy regulon in MTBC Lineage 4 [47]. Previous gene expression studies in the *in vitro* Wayne model with *M. tuberculosis* also reported similar findings [48–49]. Investigating the expression of *M. africanum* L6 genes following growth under the Wayne model for instance should provide further insights into differences in the response of *M. africanum* L6 and *M. tuberculosis* lineages to low oxygen.

Taken together, our results indicate that *M. africanum* L6 is less reliant on DosR signaling, and might pursue a different survival strategy within the human host than *M. tuberculosis* L4. Assuming that *M. africanum* L6 and *M. tuberculosis* L4 both infect the same host tissues, a loss of DosR regulon activity could be due to a DosR-independent adaptation and overall preference of *M. africanum* L6 to hypoxic, or even, anaerobic growth. Supporting this is a recent study that described an association of *M. africanum* L6 and extrapulmonary disease, reflective of an anaerobic niche [8]. Given that transmission to new hosts depends on the development of pulmonary disease, the evolutionary advantage of extrapulmonary disease is not clear. While *M. africanum* L6 is as transmissible as *M. tuberculosis* L4 from pulmonary TB patients to their contacts [5], we postulate that a relatively larger reservoir of latent and/or extrapulmonary infection by *M. africanum* L6 may offer a degree of protection against re-infection with the more virulent *M. tuberculosis* L4, maintaining *M. africanum* L6 endemicity in West Africa.

5. Conclusion

Using *ex vivo* sputum expression data, we show for the first time directly in sputum samples from patients with TB that the DosR regulon was significantly less expressed in *M. africanum* L6 compared to *M. tuberculosis* L4. We describe a clinically relevant lineage, *M. africanum* L6, which appears to have adapted to growth under hypoxic conditions or different biological niches. We provide gene expression, phenotypic and sequencing data supporting this. *M. africanum* L6 permits to study factors that have contributed to the virulence and success of the MTBc, which could be exploited to target further attenuation. Such studies will improve understanding of additional biologically relevant differences between *M. tuberculosis* and *M. africanum*.

Such a comparison is also justified by the consideration of the DosR regulon as potential vaccine- and drug target, aiming to curtail mycobacterial survival. Over the last decade, *M. tuberculosis* respiration and energy metabolism has been targeted in TB drug discovery with success. Roles for the DosR regulon in the mechanism of action of drugs and phenotypic drug tolerance have been reported [50–51]. If we are to make greater strides in controlling this well adapted human pathogen, whether through novel therapies or vaccines, it will be essential to acquire deeper insight into the role the DosR regulon plays under different stimuli and the full spectrum of influence it has on metabolism and disease development by all the different MTBc lineages. The imminent question is whether the DosR regulon will be a viable target in all MTBc strains. This remains to be answered. Understanding strain differences in more detail will facilitate the development of improved therapies useful in all TB endemic settings.

Acknowledgments

The authors thank patients and colleagues at MRC for their contribution to the present analysis. This work was supported by the European Research Council-INTERRUPTB starting grant nr.311725 (to BdJ, BO, FG, MA, CM).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2017.03.001.

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