

Cytological and Transcript Analyses Reveal Fat and Lazy Persister-Like Bacilli in Tuberculous Sputum

Natalie J. Garton¹, Simon J. Waddell², Anna L. Sherratt¹, Su-Min Lee¹, Rebecca J. Smith¹, Claire Senner², Jason Hinds², Kumar Rajakumar^{1,3}, Richard A. Adegbola⁴, Gurdyal S. Besra⁵, Philip D. Butcher^{2*}, Michael R. Barer^{1,3*}

1 Department of Infection, Immunity and Inflammation, University of Leicester Medical School, Leicester, United Kingdom, **2** Medical Microbiology, Division of Cellular and Molecular Medicine, St George's University of London, London, United Kingdom, **3** Department of Clinical Microbiology, University Hospitals of Leicester National Health Service Trust, Leicester, United Kingdom, **4** Medical Research Council Laboratories, Fajara, Banjul, The Gambia, **5** School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom

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Abbreviations: AFB, acid-fast bacilli; NRP, nonreplicating persistent

* To whom correspondence should be addressed. E-mail: butcherp@sgul.ac.uk (PDB); mrb19@le.ac.uk (MRB)

These authors contributed equally to this work.

ABSTRACT

Background

Tuberculous sputum provides a sample of bacilli that must be eliminated by chemotherapy and that may go on to transmit infection. A preliminary observation that *Mycobacterium tuberculosis* cells contain triacylglycerol lipid bodies in sputum, but not when growing in vitro, led us to investigate the extent of this phenomenon and its physiological basis.

Methods and Findings

Microscopy-positive sputum samples from the UK and The Gambia were investigated for their content of lipid body-positive mycobacteria by combined Nile red and auramine staining. All samples contained a lipid body-positive population varying from 3% to 86% of the acid-fast bacilli present. The recent finding that triacylglycerol synthase is expressed by mycobacteria when they enter in vitro nonreplicating persistence led us to investigate whether this state was also associated with lipid body formation. We found that, when placed in laboratory conditions inducing nonreplicating persistence, two *M. tuberculosis* strains had lipid body levels comparable to those found in sputum. We investigated these physiological findings further by comparing the *M. tuberculosis* transcriptome of growing and nonreplicating persistence cultures with that obtained directly from sputum samples. Although sputum has traditionally been thought to contain actively growing tubercle bacilli, our transcript analyses refute the hypothesis that these cells predominate. Rather, they reinforce the results of the lipid body analyses by revealing transcriptional signatures that can be clearly attributed to slowly replicating or nonreplicating mycobacteria. Finally, the lipid body count was highly correlated ($R^2 = 0.64$, $p < 0.03$) with time to positivity in diagnostic liquid cultures, thereby establishing a direct link between this cytological feature and the size of a potential nonreplicating population.

Conclusion

As nonreplicating tubercle bacilli are tolerant to the cidal action of antibiotics and resistant to multiple stresses, identification of this persister-like population of tubercle bacilli in sputum presents exciting and tractable new opportunities to investigate both responses to chemotherapy and the transmission of tuberculosis.

The Editors' Summary of this article follows the references.

Introduction

Mycobacterium tuberculosis infects one in three worldwide and kills more people each year than any other bacterial pathogen. Routine treatment of tuberculosis requires combination antibiotic therapy for a minimum of six months, and places a substantial burden on health care systems, particularly in resource-poor countries. Over eight million new cases every year testify to this obligate pathogen's ongoing success in transmission [1], yet we know little about what the organism needs to achieve this essential step.

Expectorated tubercle bacilli have been thought to originate from rapid and extensive bacterial growth at the margins of liquefied lesions in the lung [2,3]. Sputum provides a tractable sample of the bacterial population that must be targeted by antibiotic therapy and a snapshot of the organism on its way to a new host. It follows that the bacilli in microscopy smear-positive tuberculosis sputum express properties required for transmission—properties that might explain the existence of drug-tolerant persister subpopulations and account for the prolonged antibiotic therapy necessary for relapse-free treatment [4]. Since transmission is required for evolutionary survival, we may assume that *M. tuberculosis* experiences powerful selection pressures to maintain and express these as-yet unidentified properties. Thus, any bacillary phenotype recognised preferentially in sputum could provide clues to these properties.

We have previously shown that nonpathogenic mycobacteria readily accumulate intracellular triacylglycerol lipid bodies in vitro [5]; these bodies could not be demonstrated under similar conditions with *M. tuberculosis*, yet anecdotally have been seen in acid-fast bacilli (AFB) in tuberculous sputum [5]. The recent discovery of a novel class of diacylglycerol acyl transferase enzymes in *Acinetobacter* [6] and the subsequent characterisation of 15 members of this class as triacylglycerol synthase-encoding genes (*tgs1–tgs15*) in *M. tuberculosis* [7] provide a biochemical basis for the presence of lipid bodies in this organism. Intriguingly, Tgs1, the most active of these enzymes, is a member of the DosR regulon [8], a set of genes responsive to hypoxia and linked to long-term survival of *M. tuberculosis* in animal hosts [9–13]. It has recently been shown that triacylglycerol is accumulated by *M. tuberculosis* following hypoxic and other stresses [7,14] and may contribute to long-term mycobacterial survival. These observations raise the possibility that lipid body-positive cells in sputum may be in a nonreplicating persistent (NRP) state, which, given that NRP bacilli display antibiotic tolerance [11,13,15], would have implications for chemotherapy.

Defining the phenotypes of bacterial pathogens in their natural environments remains a key challenge. Accurate knowledge of the properties expressed at different stages of infection enables precise targeting of therapeutic and preventive measures. While much has been learnt about bacterial pathogens from in vitro and in vivo (animal model) transcriptome studies [16,17] as well as from human lung tissue [18], there have, to the best of our knowledge, been no published studies of transcript profiles in sputum samples—a clinically tractable sample. Such methods as rapidly stabilised RNA, differential cell lysis, and RNA amplification have enabled us to report here the transcriptome of *M. tuberculosis* in the sputum of patients prior to treatment.

Methods

Patients

Patients attending the public clinic at the MRC Laboratories, Fajara, The Gambia and identified as sputum smear-positive by routine microscopy were invited to provide early-morning samples for transcriptome analysis. Patients who agreed to participate gave informed oral consent (study nos. L2002.52 and L2006.60, ethical committee, MRC Laboratories, Fajara, The Gambia). Sputum from nine patients yielded sufficient mycobacterial RNA for analysis by microarray or PCR; these were designated sputum samples 1–9.

Mycobacterial Strains and Growth Conditions

M. tuberculosis complex for direct microarray transcriptome analysis was isolated from an aliquot of sputum 1 using standard methods [19]. *M. tuberculosis* complex was grown on 7H10 agar with oleic acid-albumin-dextrose-catalase [20] supplement or in 7H9 broth with albumin-dextrose-catalase supplement [20], 0.2% glycerol and 0.05% Tween-80. For hypoxic (nonreplicating persistence) cultures *M. tuberculosis* strains H37Rv and CH [21] were grown in Dubos Tween-albumin broth.

Routine Culturing of Smear-Positive Sputum Samples

Diagnostic sputum specimens were stained with auramine-phenol [19], and positive smears confirmed and scored by Ziehl-Neelsen staining after initial examination by fluorescence microscopy. Smears were scored as either 1+ (1–10 AFB in 100 fields of view), 2+ (1–10 AFB in ten fields of view), or 3+ (1–10 AFB in one field of view). Decontamination of specimens was performed by the NaOH-NALC method [19]. Each decontaminated specimen was inoculated into one vial of BACTEC 9000 MB medium for isolation of *M. tuberculosis*. The time to positivity of the BACTEC culture was recorded in days. All mycobacterial cultures were identified and confirmed as *M. tuberculosis* complex using standard procedures.

Auramine-Nile Red Labelling of Sputum Samples

Whole sputum (~1–4 ml) was digested for 15 min with an equal volume of 0.5% w/v *N*-acetyl L-cysteine in 50 mM sodium citrate [19]. Phosphate buffer (67 mM [pH 6.8]) was added to a final volume of 20 ml, and bacteria were concentrated (1,398g, 20 min). The pellet was resuspended in 0.5 ml of phosphate-buffered saline and a smear prepared with ~10 µl of the suspension. Heat-fixed smears were labelled with auramine-Nile red as previously described [5]. Preparations were observed by epifluorescence microscopy using a Nikon Diphot 300 inverted microscope with a 100 W mercury light source. Images were recorded using a 12/10bit, high speed Peltier-cooled CCD camera (FDI, Photonic Science) using Image-Pro Plus (Media Cybernetics) software. The 11001V2 Blue (excitation 470 ± 40 nm; emission > 515 nm; Chroma Technology) and the G-2A (excitation 510–560 nm; emission: 590 ± 10 nm, Nikon) filter sets were used for epifluorescence microscopy.

Nile Red Labelling of Nonreplicating Persistence *M. tuberculosis* Cultures

M. tuberculosis H37Rv and strain CH [21] were grown as agitated, aerated cultures (370 rpm) to mid-log phase in Dubos liquid medium, supplemented with Dubos medium albumin. *M. tuberculosis* NRP1/2 (nonreplicating persistence

stage 1 and 2) cultures were incubated with continuous stirring at 37 °C for 168, 288, and 504 h, respectively, according to Wayne and Hayes [15]. At these time points one tube of each culture was destructively sampled for microscopic analysis. 10 µl of each sample was spread on a slide, heat fixed, and labelled with Nile red as previously described [5].

RNA Extraction from Tuberculous Sputa

With the exception of sputum sample 1, which was frozen in liquid nitrogen within 10 min of expectoration, approximately four volumes of GTC solution (5 M guanidinium thiocyanate, 0.5% w/v sodium N-lauryl sarcosine, 25 mM trisodium citrate, 0.1 M 2-mercaptoethanol, 0.5% w/v Tween 80 [pH 7.0]) [22] were added to sputum within 5 min of collection. Mycobacteria were harvested by centrifugation (1398g, 30 min), resuspended in 400 µl of sterile deionized water, and added to 1 ml of Trizol LS (Invitrogen). RNA was extracted using a method modified from that of Desjardin et al. [23] with chloroform replacing chloroform:isoamyl alcohol washes and the Cleanascite step omitted. The mixture was transferred to a glass matrix tube for cell lysis (Lysing matrix B; Q-Biogene) and processed in a spin/rotation instrument for cell lysis (Ribolyser; Hybaid), with a speed setting of 6.5 and a time setting of 45 s. After processing, 200 µl of chloroform was added to the mixture and it was vortex-mixed for 2 min. The aqueous and organic layers were separated by microcentrifugation for 15 min at room temperature at 16,000g. The aqueous phase containing the RNA was washed once with an equal volume of chloroform. The aqueous phase was removed to a fresh tube and 1 µl of glycoblue (Ambion), 0.1 volume of 5 M ammonium acetate, and an equal volume of isopropanol were added. The RNA was precipitated overnight at -20 °C. The resulting RNA pellet was washed once with 70% v/v and once with 95% v/v ethanol, dried and resuspended in 100 µl of RNase-free H₂O (Sigma). The cognate *M. tuberculosis* complex isolate from sputum sample 1 was cultured for 6 d in 100ml 7H9 broth at 37 °C, 200 rpm at which time the absorbance was 0.22 at 580 nm. Mycobacterial RNA was stabilised with GTC solution and extracted as previously described. Total RNA from sputum samples (5, 7, 8, and 9) for amplification was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyser (Agilent Technologies).

Microarray Analysis of RNA from Sputum 1

RNA from sputum 1 and the in vitro-grown cognate isolate was cleaned using the RNeasy kit (Qiagen). A *M. tuberculosis* whole genome microarray, generated by the Bacterial Microarray Group at St. George's (University of London) and consisting of 3,924 gene-specific PCR products (designed with minimal cross-homology) to the *M. tuberculosis* H37Rv [24], was utilised (ArrayExpress accession number A-BUGS-1; <http://bugs.sgu.ac.uk/A-BUGS-1>). Hybridisations were conducted as previously described [25] with 15 µg of Cy5-labelled cDNA derived from *M. tuberculosis* RNA against 1 µg Cy3-labelled *M. tuberculosis* H37Rv genomic DNA. The hybridised slides were scanned sequentially at 532 nm and 635 nm corresponding to Cy3 and Cy5 excitation maxima using the Affymetrix 428 Array Scanner (MWG). Comparative spot intensities from the images were calculated using ImageJ 5.5 (BioDiscovery), and imported into GeneSpring GX 7.2

(Agilent Technologies) for further analysis. After local background subtraction the measured intensity in the cDNA channel for each gene was divided by its intensity in the genomic DNA control channel. The array data were normalised to the 50th percentile of all genes detected to be present on the array and filtered to remove unreliable low intensity data (below a value of 500 in either channel). Genes were identified as differentially expressed in sputum with a cut-off of >3-fold relative to in vitro growth.

Growth Conditions and RNA Extraction for Microarray Analysis

M. tuberculosis H37Rv was grown as agitated, aerated cultures (370 rpm) to mid-log phase at 37 °C in Dubos liquid medium, supplemented with Dubos medium albumin. *M. tuberculosis* NRP1/2 cultures were set up and cultured in a stirred model for 72 h and 240 h, respectively, according to Wayne and Hayes [15]. Mycobacterial RNA was extracted from in vitro models (collected straight into GTC solution) using the GTC/Trizol method as developed by Mangan et al. [26]; RNA was DNase-treated and purified using RNeasy columns (Qiagen). Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyser (Agilent Technologies).

RNA Amplification

An aliquot of 5 ng of total *M. tuberculosis* RNA was amplified using an Eberwine T7-oligo-dT based system after an initial polyadenylation step (MessageAmp II Bacteria, Ambion). Using this method, bacterial RNA was polyadenylated before priming the first-strand cDNA synthesis reaction with T7-linked oligo-dT. Amplified RNA was generated after second-strand cDNA synthesis and cDNA purification by in vitro run-off transcription (IVT) using T7 polymerase. Single rounds of amplification were performed, with an in vitro transcription reaction of 16 h at 37 °C. This amplification method has been previously demonstrated to be reproducible and capable of identifying representative changes in gene expression [27,28]. The yield and size distribution of amplified products was assessed spectrophotometrically at OD₂₆₀ and using the Agilent 2100 Bioanalyser (Agilent Technologies).

Microarray Analyses of Samples 5, 7, 8, and 9

An *M. tuberculosis* whole-genome microarray, generated by the Bacterial Microarray Group at St. George's (ArrayExpress accession number A-BUGS-23; <http://bugs.sgu.ac.uk/A-BUGS-23>), and consisting of 4,410 gene-specific PCR products (designed with minimal cross-homology) to the *M. tuberculosis* H37Rv [24], CDC1551 [29], and *M. bovis* AF2122/97 [30] genomes was utilised. Hybridisations were conducted as previously described [25] except for the use of *M. tuberculosis* genomic DNA as a common reference [31]. Using genomic DNA reduced technical variation between replicate hybridisations and allowed RNA profiles to be used in multiple comparisons. 5 µg of Cy5-labelled cDNA derived from amplified *M. tuberculosis* RNA was hybridised with 2 µg of Cy3-labelled *M. tuberculosis* H37Rv genomic DNA. A lower ratio of test cDNA to comparator gDNA was used than with sample 1, as we were able to confirm the purity of our preparations with the Bioanalyser at the same time and perform technical replicates. The *M. tuberculosis* H37Rv

reference DNA was kindly provided by Colorado State University (<http://www.cvmbs.colostate.edu/microbiology/tb/top.htm>). Two biological replicates of each in vitro growth condition and four sputum samples (5, 7, 8, and 9) were hybridised in triplicate. The microarrays were scanned and spot intensities calculated as described above. The array data were normalised to the 50th percentile of all genes detected to be present on the array. The dataset was filtered to include only cDNA elements flagged to be present on 80% of the arrays. Significantly differentially expressed genes were identified using ANOVA ($p < 0.05$ with Benjamini and Hochberg multiple testing correction) and a fold change of > 2.5 . The significantly differentially expressed genes were hierarchically clustered using Cluster and the results displayed using Treeview software [32]. The hypergeometric distribution was used to determine if functional categories of genes were significantly enriched in the sputum profile [33]. Fully annotated microarray data are deposited in BμG@Sbase and ArrayExpress.

Quantitative Real-Time RT-PCR

Mycobacterial RNA (0.5 μg) from sputum sample 1 and its cognate isolate and eight further sputum samples (samples 2–9) were reverse transcribed in a total volume of 30 μl using random primers and Superscript II (Invitrogen Technologies) according to manufacturer's instructions. To estimate DNA contamination of samples, all were subjected to a no reverse transcriptase control, which was then subtracted from the RNA result. A no-reverse transcriptase threshold of 10% of the test value was taken, with the exception of four reactions in which values which were $< 20\%$ were used for correction. PCR reactions for *tgsl* were set up using Absolute QPCR SYBR green mix (ABgene), 0.4 μM primers [7] and 2 μl of cDNA. PCR was performed using the Rotor-Gene RG-3000 system (Corbett Research) heating to 56 °C for 2 min, then 95 °C for 15 min, before 40 cycles of 95 °C for 30s, 60 °C for 30s, and 72 °C for 30s, acquiring fluorescence at 85 °C. No-reverse transcriptase controls for both the sputum and isolate RNA were included, and these showed no PCR product. PCR for *icl1* was set up with the primers of Dubnau et al. [34] using the same cycling conditions and acquiring fluorescence at 86 °C. An *hspX* PCR was performed using primers of Wilkinson et al. [35] with 40 cycles of 95 °C for 30s, 59 °C for 30s, 72 °C for 30s, acquisition of fluorescence at 85 °C. For normalisation, PCR of *sigA* was performed using the primers of Manganelli et al. [36], and cycling conditions used for *tgsl* with fluorescence acquisition at 86 °C. PCRs for *nuoB*, *qcrC*, and *ctaD* were performed using primers of Shi et al. [37] with conditions as previously described with annealing steps performed at 61 °C, 56 °C, and 56 °C, and acquisition of fluorescence at 84 °C, 82 °C, and 83 °C, respectively. The quantity of target DNA in each cDNA sample was determined by the threshold cycle (C_T) with reference to a standard curve generated by the amplification of known amounts of *M. tuberculosis* H37Rv genomic DNA.

Statistics

The proportion of lipid body-positive AFB and time to positivity of routine cultures were analysed by linear regression to provide R^2 correlation coefficients. As the lipid body content of these samples was not normally distributed, Pearson correlations were also performed.

Results

Lipid Body-Positive Acid-Fast Bacilli Are a Universal Feature of Smear-Positive Tuberculous Sputum

If lipid body-positive cells are a transmission-adapted phenotype for *M. tuberculosis*, then such cells should be present in most smear-positive sputum samples. We confirm this hypothesis in 82 smear-positive samples from patients from The Gambia and the UK (69 and 13, respectively). In samples with > 100 assessable bacilli, the frequency of lipid body-positive cells varied from 3% to 86% (mean 45%, standard deviation 20%), and these contained between two and eight lipid bodies per cell (Figure 1). Thus, lipid body-positive tubercle bacilli are readily demonstrable in smear-positive samples from tuberculosis patients in two well-separated geographic locations and are present in a subpopulation of mycobacterial cells.

Lipid Bodies Are Readily Observed in *M. tuberculosis* Cells in Nonreplicating Persistence

The discovery and characterisation of *tgsl* [6,7] raised the possibility that lipid bodies might be formed in response to the hypoxic growth shift-down conditions that have been described by Wayne and Hayes [15], conditions known to cause up-regulation of the DosR regulon [9,10]. When *M. tuberculosis* H37Rv, a laboratory strain, and CH, a recent clinical isolate responsible for a large outbreak [21], were exposed in vitro to these conditions, abundant Nile red-staining lipid bodies were observed in both strains; respectively, 29% and 42% in NRP1 (168 h), 50% and 65% in NRP2 (288 h), and 41% and 56% in late NRP2 (504 h). An average of two lipid bodies per cell (range one to five) was observed in all samples except for the H37Rv NRP1 sample in which only one (range one to three) was seen in positive cells. Thus, NRP *M. tuberculosis* cultures contain lipid bodies at levels comparable to those seen in sputum.

Expression Profiling of *M. tuberculosis* Recovered from Sputum

If lipid bodies are a biomarker for cells in an NRP state, then the *M. tuberculosis* transcripts present in sputum should be compatible with those observed in NRP in vitro studies [9,10]. We therefore compared the transcriptome of *M. tuberculosis* recovered from human sputum to that obtained from in vitro aerobic cultures and NRP-inducing conditions [15]. Twenty sputa were collected from known microscopy-positive Gambian patients before they started antibiotic treatment, and the samples were rapidly stabilized against RNA degradation. Five samples (designated 1, 5, 7, 8, and 9) were analysed by microarray hybridisation, four with and one without prior polyadenylation/oligo-dT based amplification. Although the results from sputum 1, the single direct (nonamplified) array, are not discussed further, they confirm the essential details of the amplified analyses (Tables S1 and S2). This high-volume sample (~30 ml) had an exceptionally high bacterial load, and we estimate that $> 10^{10}$ bacilli were present. The data from the four amplified samples were analysed with array hybridisations of amplified RNA extracted from *M. tuberculosis* H37Rv under different conditions: log-phase aerobic growth, the two stages of NRP (NRP1 $t = 72$ h; NRP2 $t = 240$ h) [15], and a mixed preparation containing RNA from aerobic and NRP2 cells mixed in the proportion 70:30 (w/w total RNA). This latter preparation was included

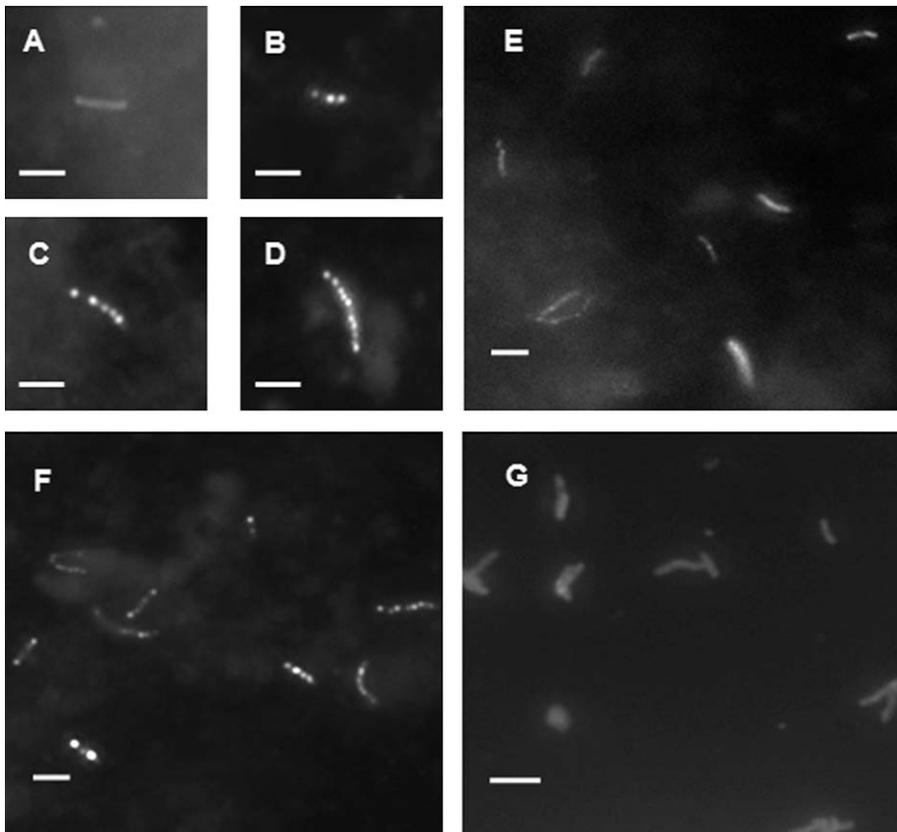


Figure 1. Lipid Bodies in Tuberculous Sputum Samples

Auramine/Nile red-fixed sputum smears [5] and aerobic *M. tuberculosis* growth. Variation in lipid bodies per cell: (A) none, (B) three, (C) five, and (D) eight. Samples are shown with (E) low and (F) high proportions of lipid body-positive cells. (G) Aerobically grown mid-log *M. tuberculosis* H37Rv contained negligible lipid bodies. Scale bar 2 μ m.
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because this mixture was representative of the lipid body-positive population in sputum. This preparation therefore enabled us to test the hypothesis that sputum comprises a mixture of the rapidly and aerobically growing bacilli expected at the margins of liquefying caseous lesions [2] with the NRP-like cells indicated by our lipid body studies.

Microarray data analysis revealed that, after filtering to remove genes with low signals in either channel, 182 genes were significantly induced in sputum compared to aerobic growth, and 334 genes were significantly repressed (Tables S3 and S4). Figure 2 displays the results of gene cluster analysis of array data from the biological and technical replicates for these genes across the sputa, NRP and mixed aerobic:NRP2 sample sets. Boxes 1 and 2 highlight gene clusters similarly regulated in NRP2 and sputum relative to aerobic growth. We note the large cluster of strongly down-regulated signals in sputum, a feature lost in box 1 in the 70:30 mix, presumably due to the aerobic signals obscuring the NRP2 signals. A similar pattern of differential expression is apparent for the amplified RNA from the four sputum samples, even though they came from separate, untreated patients.

The data show that none of our comparator conditions, including the 70:30 aerobically replicating:NRP2 mixture, closely parallel the sputum transcriptome. While significant overlaps between the genes differentially expressed in sputum were revealed by hypergeometric probability values (Tables S5 and S6), no single or obvious combination of

defined conditions herein, nor previously reported in vitro or in vivo, correspond to the signature we have obtained from sputum. Amongst the different functional categories of genes, relative to aerobic growth there were significant decreases in expression of genes required for aerobic respiration and ribosomal function and an increase in transcripts associated with cholesterol utilisation (Figure 3) [38]. We note also that genes previously observed to be repressed during bacillary stasis in a chronic murine infection model [37], *nuoB*, *ctaD*, *qcrC*, *atpA*, and *atpD*, followed this pattern in our data while *narK2* was up-regulated, as was the case in the murine studies. DosR was the most prominently activated regulon in sputum (box 2 in Figure 2; Tables S2 and S5), although the level of activation was lower than in the comparator conditions.

The induction of the isocitrate lyase gene, *icl1*, is consistent with the expected shift to utilisation of lipids as a source of carbon and energy [39]. This in vivo-associated metabolic pattern has emerged from other in vivo studies [11,12,39–42]; we particularly note the signals indicating cholesterol utilisation related to the putative KstR regulon [38,43], a feature that corresponds well with prominent sputum cholesterol content detected by thin layer chromatography (NJG, unpublished data) and the presence of this lipid in pulmonary exudates. The combination of DosR activation, lipid utilisation, and a slow growth signature is similar to experimental conditions previously studied in animal and macrophage infections [11,12], as depicted in Figures 3 and 4.

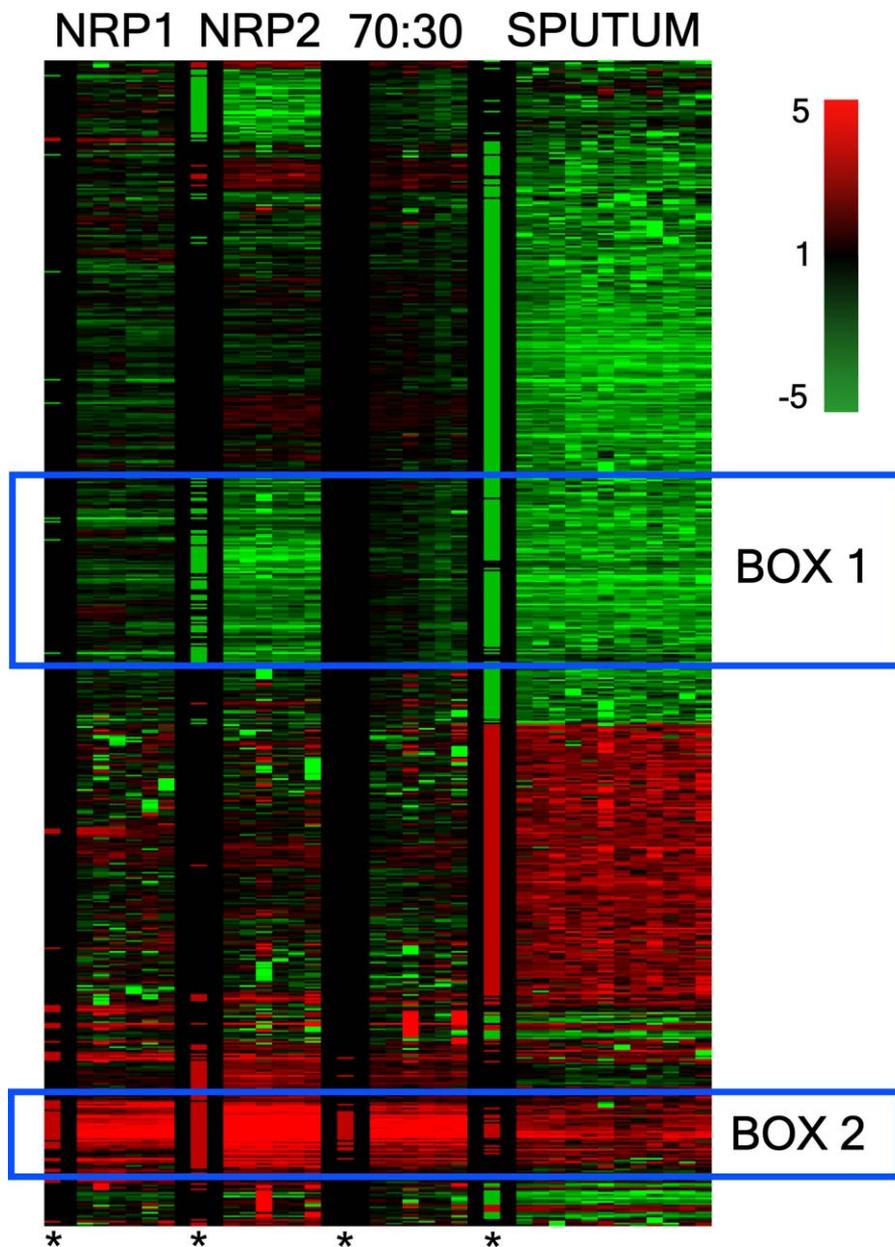


Figure 2. Display of Genes Differentially Regulated in Sputum and NRP versus Aerobic Culture

Clustering of 648 genes significantly differentially expressed in either sputum, NRP1, NRP2, or a 70:30 mix of aerobic:NRP2 compared to aerobic growth. Biological and technical replicates of conditions are displayed as columns, genes as rows. Red represents the induction of gene expression relative to aerobic growth, green repression. Asterisked columns mark the conditions in which genes were identified as significantly differentially expressed compared to aerobic growth. Boxes 1 and 2 highlight clusters of genes similarly regulated in NRP2 and sputum.
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However, induction of Fe^{2+} scavenging was absent from our dataset, presumably due to an excess of available Fe^{2+} in necrotic liquefying tissue (Tables S3–S6).

We have confirmed key features of the sputum transcriptome and its relation to the metabolic states of bacilli in sputum with selected qRT-PCR analyses applied to up- and down-regulated transcripts (Figure 5). Up-regulation of *tgsI* was detected in unamplified (Table S1) and amplified (Table S3) array analyses and qRT-PCR confirmed this in these and four further samples (designated 2–4 and 6). While the up-regulation of *iclI* confirms the shift towards lipid utilisation, the strong *hspX* (α -crystallin homologue), *narK2* (nitrate/

nitrite transporter), and *tgsI* transcript signals confirm DosR up-regulation [8]. Down-regulation of *nuoB* (type-I NADH dehydrogenase), *qcrC* (cytochrome bc_1 complex), and *ctaD* (aa_3 -type cytochrome *c* oxidase) confirms a reduction in efficiency of the aerobic respiratory chain [37].

The strong expression of *tgsI* in sputum and the presence of lipid body-positive *M. tuberculosis* cells therein suggest a likely direct link between *tgsI* expression, lipid body formation, and increased bacillary triacylglycerol content. We have demonstrated such a link in *M. smegmatis* by overexpressing *tgsI* in this organism (see Figure S1): both increased triacylglycerol and lipid body content were observed following *tgsI* induction.

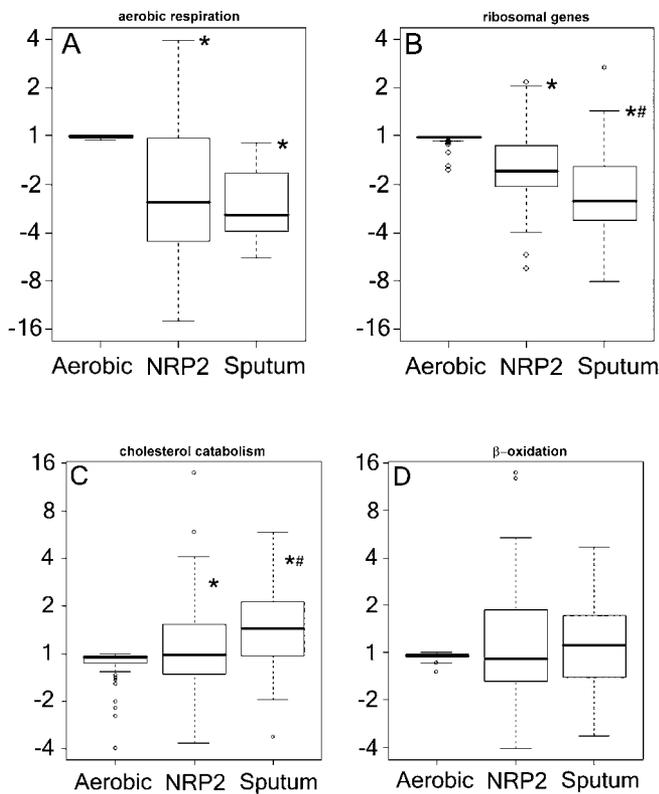


Figure 3. Genes Required for Aerobic Respiration and Ribosomal Function Show Decreased Expression in Sputum Compared with Aerobic Growth while Genes Involved in Lipid Metabolism Were Induced

Box and whisker plots showing the distribution of expression ratios (log2 scale) of (A) 21 aerobic respiration genes and (B) 45 ribosomal genes in NRP2 and sputum relative to aerobic growth using functional classifications defined by Cole et al., 1998 [24]; also (C) 64 genes that may be involved in cholesterol catabolism [38] and (D) 45 genes in the *fadB*, *echA*, *fadE*, and *fadA* families, which may be involved in the β -oxidation of fatty acids. *Significant difference ($p < 0.01$) between NRP2 or sputum compared to aerobic; #Significant difference ($p < 0.01$) between NRP2 and sputum.

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The Frequency of Lipid Body-Positive Acid-Fast Bacilli in Sputum Is Correlated with “Time to Positivity” in Routine Diagnostic Liquid Culture

Although both the lipid body and the transcriptome results are consistent with the presence of a NRP-like population in sputum, more direct evidence that the lipid body-positive cells have the properties one might expect of cells in this state would be desirable. Standard bacteriology tells us that nonreplicating bacterial cells take longer to initiate growth than their replicating counterparts (longer lag phase) [44,45]. If the lipid body-positive cell count provides an estimate of a NRP population in sputum, then this should be reflected in the “time to positivity” in liquid culture. Figure 6 demonstrates that “time to positivity” is significantly associated with lipid body percentage in 15 diagnostic samples with $p < 0.03$ and $R^2 = 0.64$.

Discussion

We applied a combination of ex vivo and in vitro analyses to study the phenotypes of *M. tuberculosis* cells in smear-positive sputum samples. We found a subpopulation of lipid

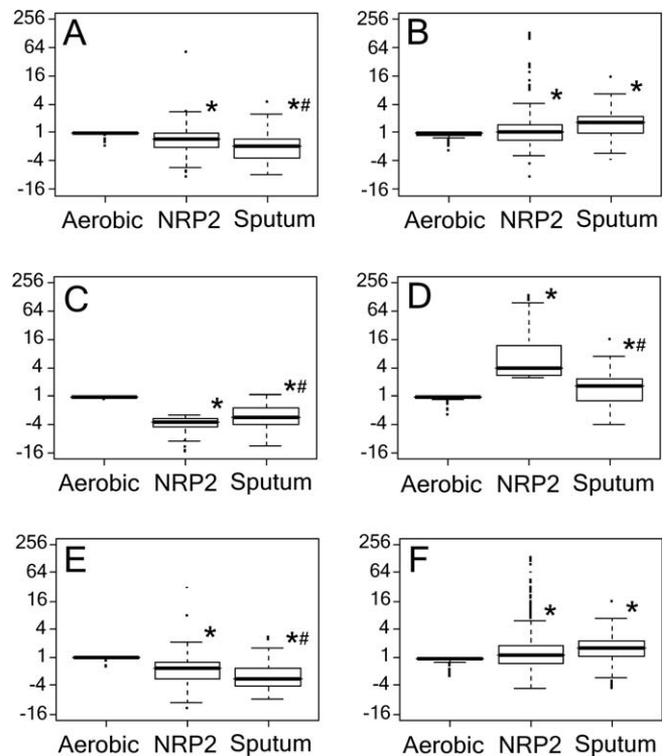


Figure 4. Gene Expression Signatures Representative of Slow Growth and the *M. tuberculosis* In Vivo Phenotype Were Identified in the Sputum Transcriptome

The distribution of expression ratios (log2 scale) of (A) 129 genes repressed and (B) 127 genes induced by slow growth [53]; (C) 106 genes repressed and (D) 85 genes induced by NRP2 compared to aerobic growth (this report); (E) 111 genes repressed and (F) 339 genes induced on murine macrophage infection [12]. In all plots the y-axis denotes fold change, boxes encompass the 25th and 75th percentiles, whiskers have been set at 1.5 \times the range between these values, and only outliers are shown as individual points. *Significant difference ($p < 0.01$) between NRP2 or sputum compared to aerobic; #Significant difference ($p < 0.01$) between NRP2 and sputum.

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body-positive acid-fast cells in all samples for which >100 bacilli were analysable. Our further in vitro studies revealed nonreplicating persistence, as defined by Wayne and colleagues [46], to be a condition in which *M. tuberculosis* cells are induced to form lipid bodies at frequencies comparable to those observed among tubercle bacilli in sputum. Consistent with this finding, transcriptome analysis of *M. tuberculosis* in sputum revealed signals compatible with slow or non-growth and absence of aerobic respiration. Moreover, the time to positivity in diagnostic liquid culture was shown to be directly related to sputum lipid body content, adding further weight to the view that lipid body-positive cells are not replicating. While other explanations remain possible, we conclude that the lipid body-positive cells in sputum have a persister-like phenotype, with important implications for the treatment and transmission of tuberculosis. Further studies should elucidate the impact of chemotherapy on the frequency of lipid body-positive populations of *M. tuberculosis* in patient sputum, and the relationship between this candidate biomarker and both infectivity and the clinical response to treatment.

The analysis of tuberculous sputum has played a central

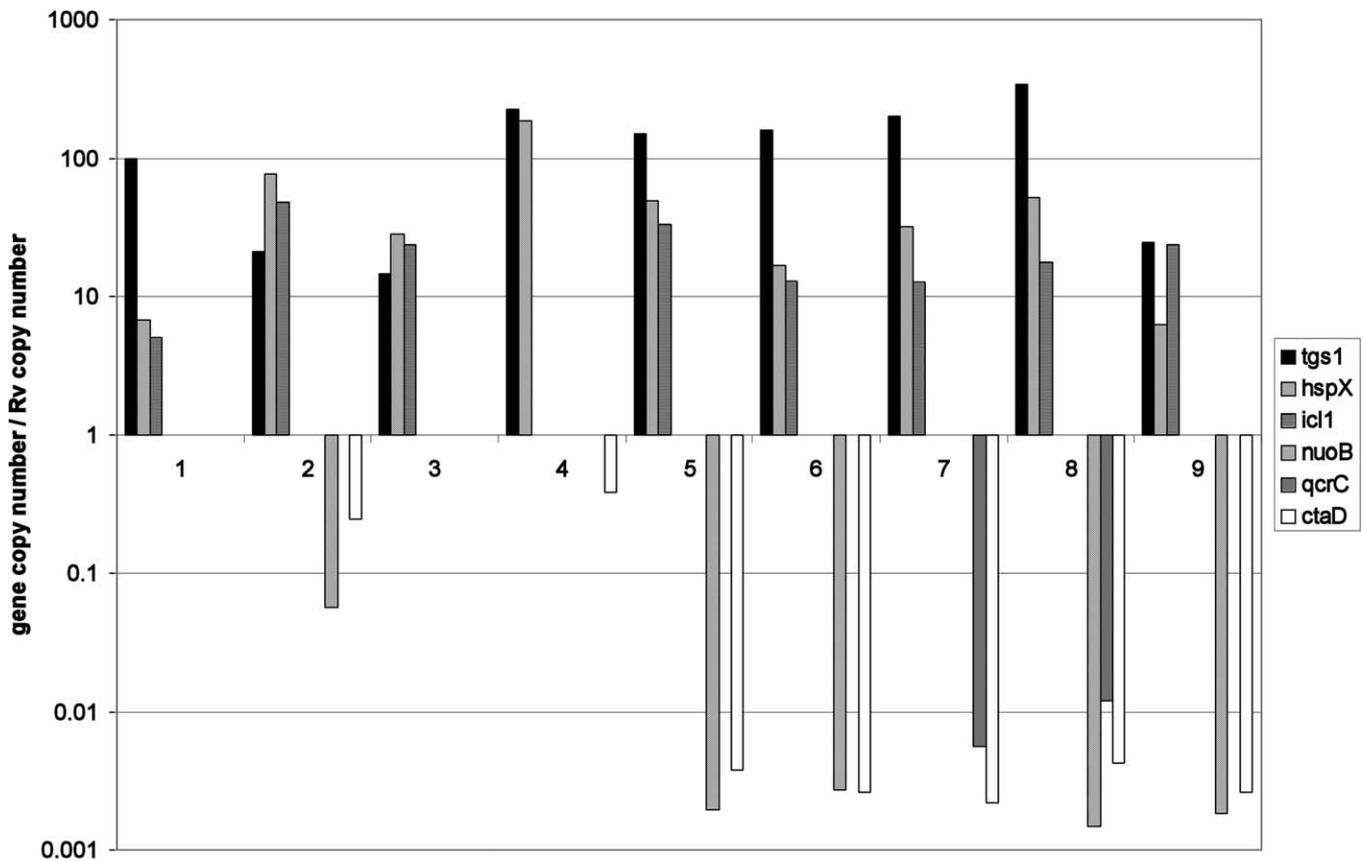


Figure 5. Specific Transcript Ratios for *tgs1*, *hspX*, *icl1*, *nuoB*, *qcrC*, and *ctaD* in AFB-Positive Sputum Samples Determined by qRT-PCR and Normalized to Values for Aerobically Grown Mid-Log *M. tuberculosis* H37Rv

Individual target gene transcript copy numbers were normalized against transcript copy numbers of *sigA* in the samples concerned. Numbers on the abscissa refer to the designated sputum sample numbers. doi:10.1371/journal.pmed.0050075.g005

role in the diagnosis and management of tuberculosis. While the presence of acid-fast bacilli in sputum is the feature most prominently linked to the potential of a patient to disseminate infection, there are other influential factors. Setting aside those associated with human behaviour and the immediate atmospheric conditions, a transmitted tubercle bacillus must survive transit and master new environmental pressures if it is to establish infection in a new individual. From what we know about bacterial adaptation, it is highly probable that specific traits are expressed to achieve this ability. Furthermore, it is recognised that in the treatment of tuberculosis and other bacterial infections, bacterial burden correlates not only with increased potential for onward transmission, but also with the duration of chemotherapy required for a cure [47]. Bacterial populations often show heterogeneous properties. The presence of a slow or non-growing subpopulation of bacteria phenotypically resistant to antibiotics has been proposed to account for the extended time required for treatment of tuberculosis [4,48]. Although never directly identified, the presence of such a population is inferred from the biphasic reduction of viable bacterial counts recovered from serial sputum samples collected during therapy [4]. Such antibiotic-tolerant “persister” populations have been recognised in many bacterial infections [49]; a greater bacterial burden being associated with a higher frequency of phenotypic resistance. The results we

present here are a first step towards defining the transmission phenotype of *M. tuberculosis* and also reveal directly, to our knowledge for the first time, a substantial population of persister-like bacilli in sputum prior to commencement of therapy.

Lipid bodies must now be recognised as a universal feature of smear-positive tuberculosis, and the significance of this finding and of the variation in the proportion of positive cells between samples must be established. While lipid bodies are a well-established feature of eukaryotic cell biology [50], their recognition in prokaryotes is relatively recent [51]. The link between *tgsI*, the DosR regulon, the hypoxia-induced NRP state, and lipid bodies that is strengthened and made clinically relevant by our findings, relates these structures to a coherent set of laboratory studies. Lipid body-positive cells must now be factored into the debate about mycobacterial dormancy and persistence. Fourteen functional Tgs enzymes that are not DosR regulated have been identified [7]. However, none of the mRNAs encoding these enzymes was found to be up-regulated in our transcriptional studies, while *TgsI*, the most active enzyme, and the DosR regulon itself were.

While the microarray results can be analysed in several different contexts, we focus here on the data that have a bearing on the growth state and lipid body content of our samples. The transcriptome clearly shows that the sputum

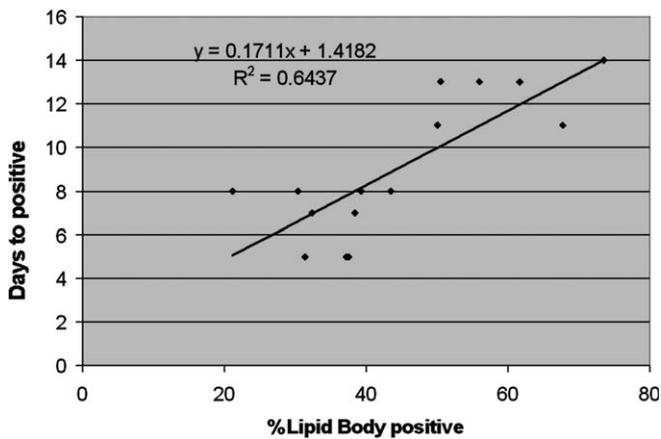


Figure 6. Time to Positivity in BACTEC 960 Cultures Related to Lipid Body Counts Determined in the Samples from Which the Cultures Were Prepared. Analysis was confined to samples graded 3+ by microscopy to minimize the effect of varying bacterial inoculum on time to positivity. doi:10.1371/journal.pmed.0050075.g006

bacillary population is dominated by slowly or nonreplicating bacilli, a contention further supported by two lines of comparative evidence. Firstly, two *in vitro* transcriptome datasets can be robustly argued to represent nonreplicating cell populations: the nutrient deprivation studies of Betts et al. [52] and our NRP2 results. Of the repressed 33 genes common to both of these datasets, 20 were found to be down-regulated in our sputum samples (hypergeometric p -value 2.56×10^{-11}), a feature that is further supported by strong correlations with the recently published reduced growth rate dataset (Figure 5) [53]. Secondly, Shi and colleagues studied specific gene expression in chronic mouse infections [37] under conditions in which there is clear evidence for lack of replication [54]. In common with this study, our results show repression of *nuoB*, *ctaD*, *qcrC*, *atpA*, and *atpD* and up-regulation of *narK2*. This supports the view that our sputum samples contained many nonreplicating bacilli in respiratory state III defined by these authors [37], that is, a shift from oxygen electron transfer to anaerobic electron transfer.

If the frequency of lipid body-positive mycobacteria in sputum provides an estimate of the NRP cells present, then other NRP-related features should be correlated. Remarkably, we found this to be the case with time to positivity in routine diagnostic cultures performed on samples that we had analysed for their lipid body content (Figure 6). These results provide direct evidence that the frequency of lipid body-positive cells provide an estimate of the nonreplicating mycobacterial population in sputum in these samples.

Drawing all these results together, we now reject the commonly held belief that smear-positive sputum is dominated by aerobically replicating *Mycobacterium tuberculosis*. The transcriptome data in particular show that such cells could only be a minor component in the samples analysed in this way. In contrast, we conclude that our samples contained nonreplicating mycobacteria at levels proportional to the lipid body-positive cells therein.

While the significance of this finding to clinical tuberculosis will only be established by long-term studies, several important implications can be recognised at this stage. First, it is clear that the large numbers of tubercle bacilli observed

in sputum are not a direct sample from extensive and rapid aerobic growth at the margins of open cavities. Rather, we propose that, as with all growth in restricted environments, this aerobic growth results in the buildup of larger and larger numbers of stationary phase nonreplicating bacilli and that this accords with the mature “colony-like” growth of tubercle bacilli reported in caseous lesions by Canetti [2]. Second, the cidal action of many antibiotics is proportional to the growth rate of bacteria, with those growing slowly or in a non-replicating state showing phenotypic tolerance [49,55,56]. In particular, Wayne type *M. tuberculosis* NRP cultures are tolerant to isoniazid and rifampin [15]. Interestingly, while such cultures provide the closest available transcriptome match to the signals we have obtained from sputum, our own studies on phenotypic resistance have so far consistently shown that the presence of *M. tuberculosis* lipid bodies accumulated following growth-arresting stimuli, is correlated with tolerance to the cidal action of these antibiotics (see Figure S2). Such phenotypic resistance is widely believed to underpin the persister phenomenon in tuberculosis, in which a residual and antibiotic-recalcitrant population requires extended chemotherapy for its elimination [4].

We emphasise that hypoxia is not the only stress capable of inducing lipid body formation. This is exemplified by our preliminary nitric oxide data (Figure S2). This latter effect is probably mediated via DosR [57]. The relationship between DosR induction and growth rate is clearly multifactorial, with the up-regulation of DosR perhaps a general indicator of mycobacterial stress, for example the DosR regulon is induced during the exponential phase of growth in mice [31]. It is the slow/nongrowth transcriptional profile and our time-to-positivity results that indicate the presence of a nonreplicating population in sputum rather than *dosR* expression, which is found in both growing and nongrowing populations. However, it should be noted that bacterial populations are evidently nonuniform. Thus lipid body-positive cells may also represent a slower or nonreplicating population within a growing culture.

We cannot say whether the expectorated persister-like population we report here reflects the persister population revealed during chemotherapy. Initial establishment of persisters in growing populations is probably random and at a low level; however, during infection these populations will be influenced by specific conditions, including the development of colonial/biofilm-like growth [2] and inflammatory responses that may increase the numbers of persister-like cells observed [49,57]. We note that all the sputum samples we examined were collected prior to the commencement of chemotherapy; the status of bacilli within patients treated with antibiotics is not clear. Nonetheless, this question is amenable to further study through the analysis of the responses of patients to therapy and serial analyses of the lipid body content of their sputum samples.

Finally, returning to the proposal that the bacilli in sputum display traits that underpin the transmission of tuberculosis, the relative resistance of nonreplicating bacteria, including *M. tuberculosis*, to a variety of stresses is well established [59–61]. Global stress resistance will promote survival that is essential for transmission. More specifically, we note that formation of lipid bodies in *Rhodococcus*, another actinomycete, has been linked to improved survival during desiccation [62]. Even more intriguing is the observation that

hypoxically grown *M. tuberculosis* cultures, in which we have demonstrated ~34% lipid body-positive cells (unpublished data), are 10-fold more infectious for guinea pigs by the aerosol route than their aerobically grown counterparts [63]. Moreover, recent investigation of Beijing strains of *M. tuberculosis* revealed that they accumulate more triacylglycerol and express *tgs1* at levels 10-fold higher than laboratory strains, during aerobic log-phase growth [64]. The enhanced transmissibility, evidenced by the rapid global spread of these strains, may reflect a greater propensity for lipid body formation in vivo.

We propose that lipid body positive (fat) acid fast bacilli are a biomarker for nonreplicating (lazy) *M. tuberculosis* cells in sputum; their further study offers exciting and tractable avenues for research into the treatment and prevention of tuberculosis.

Supporting Information

Figure S1. Overexpression of *tgs1* in *M. smegmatis* Leads to Enhanced Accumulation of Triacylglycerol and Lipid Bodies

We cloned *tgs1* under the control of the acetamide-inducible promoter pSD26 [65] and expressed it in *Mycobacterium smegmatis*, which readily forms triacylglycerol (TAG) lipid bodies in vitro. Test and control cells were exposed to radiolabelled oleic acid for 10 min and incorporation into TAG determined [66].

(A) Triacylglycerol synthase (TGS) activity of induced MspSD26-*tgs1* and MspSD26 vector control, $p < 0.001$.

(B) Pseudo-coloured fluorescence images of induced MspSD26 vector control (i) and MspSD26-*tgs1* (ii) incubated with 630 μM oleic acid for 10 min and labelled with Nile red [5]. Pseudo-colour (blue/min to red/max) was applied to grey levels 101–255 to demonstrate enhanced lipid body formation in the *tgs1*-overexpressing cells. Scale bar = 2 μm .

Found at doi:10.1371/journal.pmed.0050075.sg001 (6.7 MB TIF).

Figure S2. Nitric Oxide Exposure Stimulates Lipid Body Formation and Tolerance to the Cidal Action of Rifampin

Exponential phase *M. tuberculosis* H37Rv cultures in Sauton's medium (~ 10^5 cfu/ml) were treated with 100 μM spermine.NO (NO donor) for 4 or 24 h. These test cultures respectively contained 65% and 22% lipid body-positive cells, while the control cultures, exposed to 100 μM spermine.HCl for the same times, contained < 1%. Subsequent exposure of test and control cultures to rifampin (1 $\mu\text{g}/\text{ml}$) for 7 d revealed diminished killing in the NO exposed cultures. Error bars = + 1 standard deviation ($n = 3$).

Found at doi:10.1371/journal.pmed.0050075.sg002 (194 KB TIF).

Table S1. Genes Determined to Be Greater than 3-fold Up-regulated in Sputum 1 Compared with In Vitro Growth of the Cognate Isolate by Direct Microarray

Found at doi:10.1371/journal.pmed.0050075.st001 (52 KB DOC).

Table S2. Hypergeometric Probability Analysis of Genes Determined to be > 3-Fold Up-regulated in Sputum 1 Compared to In Vitro Cognate Isolate Growth by Direct Microarray

Found at doi:10.1371/journal.pmed.0050075.st002 (85 KB DOC).

Table S3. Genes Determined to be Greater than 2.5-Fold Up-regulated in Sputum Compared with In Vitro Growth by Amplified Microarray

Found at doi:10.1371/journal.pmed.0050075.st003 (403 KB DOC).

Table S4. Genes Determined to be Greater than 2.5-fold Down-regulated in Sputum Compared with In Vitro Growth by Amplified Microarray

Found at doi:10.1371/journal.pmed.0050075.st004 (664 KB DOC).

Table S5. Hypergeometric Probability Analysis of Genes Determined to be >2.5-Fold Up-regulated in Sputum Compared to In Vitro H37Rv Growth by Amplified Microarray

Found at doi:10.1371/journal.pmed.0050075.st005 (104 KB DOC).

Table S6. Hypergeometric Probability Analysis of Genes Determined to be >2.5-Fold Down-regulated in Sputum Compared to In Vitro H37Rv Growth by Amplified Microarray

Found at doi:10.1371/journal.pmed.0050075.st006 (86 KB DOC).

Accession Numbers

The fully annotated microarray data from this study are deposited in BμG@Sbase (accession number: E-BUGS-52; <http://bugs.sgul.ac.uk/E-BUGS-52>) and ArrayExpress (accession number: E-BUGS-52; <http://www.ebi.ac.uk/arrayexpress/experiments/E-BUGS-52>).

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Author contributions. NJG, SJW, RAA, PDB, and MRB designed the study, analysed results and contributed to the writing of the paper. KR and GSB contributed to the analysis of results and writing of the paper. NJG, SJW, ALS, SML, RJS, CS, and JH were involved in performing the experiments and analysing the data.

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Editors' Summary

Background. Every year, nearly nine million people develop tuberculosis—a contagious infection usually of the lungs—and about two million people die from the disease. Tuberculosis is caused by *Mycobacterium tuberculosis*, bacteria that are spread in airborne droplets when people with the disease cough or sneeze. The symptoms of tuberculosis include a persistent cough, weight loss, and night sweats. Diagnostic tests include chest X-rays, the tuberculin skin test, and sputum analysis. For the last of these tests, a sample of sputum (mucus and other matter brought up from the lungs by coughing) is collected and then taken to a laboratory where bacteriologists look for *M. tuberculosis* using special stains—tuberculosis-positive sputum contains “acid-fast bacilli”—and also try to grow bacteria from the sample. Tuberculosis can be cured by taking several powerful antibiotics for several months. It is very important that this treatment is completed to ensure that all the *M. tuberculosis* bacteria in the body are killed and to prevent the emergence of drug-resistant bacteria.

Why Was This Study Done? Strenuous efforts are being made to reduce the global burden of tuberculosis but with limited success so far for many reasons. One barrier to success is the efficiency with which *M. tuberculosis* spreads from one person to another. Very little is known about this part of the bacteria's life cycle. If scientists could understand more about the transmission of *M. tuberculosis* between people, they might identify new therapeutic and preventative targets. In the study, therefore, the researchers examine the acid-fast bacilli in tuberculosis-positive sputum samples to get a snapshot of *M. tuberculosis* at the point of its transmission to a new person and ask how the characteristics of these bacilli compare with those of *M. tuberculosis* growing in the laboratory.

What Did the Researchers Do and Find? The researchers collected sputum samples from patients with tuberculosis in the UK and The Gambia before they received any treatment, and looked for the presence of acid-fast bacilli containing “lipid bodies.” These small structures contain a fat called triacylglycerol. *M. tuberculosis* accumulates triacylglycerol when it is exposed to several stresses present during infection (for example, reduced oxygen or hypoxia) and the researchers suggest that the presence of this fat may help the bacteria survive during transmission and establish a new infection. They found that all the samples contained some lipid body-positive acid-fast bacilli. Next, the researchers showed that *M. tuberculosis* grown in the laboratory under hypoxic conditions, which induce the bacteria to enter an antibiotic-tolerant condition called a “nonreplicating persistent” (NRP) state, also accumulated lipid bodies. This result suggests that the lipid body-

positive acid-fast bacilli in sputum might be in an NRP state. To test this idea, the researchers compared the pattern of mRNAs (the templates from which proteins are produced; the pattern of mRNAs is called the transcriptome and gives an idea of which proteins a cell is making under given conditions) made by growing cultures of *M. tuberculosis*, by *M. tuberculosis* maintained in the NRP state, and by the acid-fast bacilli in several sputum samples. The transcriptome of the sputum sample revealed production of many proteins made in the NRP state. Finally, the researchers showed that the time needed to grow *M. tuberculosis* from sputum samples increased as the proportion of lipid body-positive acid-fast bacilli in the sputum increased, just as one would suspect if the presence of lipid bodies signifies nongrowing cells.

What Do These Findings Mean? It has been generally assumed that the acid-fast bacilli in sputum collected from patients with tuberculosis are rapidly replicating *M. tuberculosis* released from infected areas of the lungs. By identifying a population of bacteria that contain lipid bodies and that are in an NRP-like state in all the samples of sputum examined from two geographical sites, this study strongly challenges this assumption. The characteristics of this population of bacteria, the researchers suggest, might help them survive the adverse conditions that *M. tuberculosis* encounters during transmission between people and might partly explain why complete clearance of *M. tuberculosis* requires extended treatment with antibiotics. To establish the clinical significance of these findings, future studies will need to examine whether antibiotic treatment affects the frequency of lipid body-positive *M. tuberculosis* bacteria in patients' sputum and whether there is any relationship between this measurement and infectiousness, or clinical response to treatment.

Additional Information. Please access these Web sites via the online version of this summary at <http://dx.doi.org/10.1371/journal.pmed.0050075>.

- The MedlinePlus encyclopedia contains pages on tuberculosis and on sputum culture (in English and Spanish)
- The US National Institute of Allergy and Infectious Diseases provides information on all aspects of tuberculosis
- The US Centers for Disease Control and Prevention Division of Tuberculosis Elimination provides several fact sheets and other information resources about tuberculosis
- The World Health Organization provides information on efforts to reduce the global burden of tuberculosis

