Correlates between Models of Virulence for *Mycobacterium tuberculosis* among Isolates of the Central Asian Lineage: a Case for Lysozyme Resistance Testing?

Claire Pardieu,^a Nicola Casali,^{a,b} Simon O. Clark,^c Richard Hooper,^d Ann Williams,^c Preya Velji,^a Ximena Gonzalo,^{a,b} Francis Drobniowski^{a,b,e}

Clinical TB and HIV Group, Centre for Immunology and Infectious Disease, Queen Mary University of London, London, United Kingdom^a; Department of Infectious Diseases and Immunity, Hammersmith Hospital, Imperial College, London, United Kingdom^b; Porton Down, Public Health England, Salisbury, United Kingdom^c; Centre for Primary Care and Public Health, Queen Mary University of London, London, United Kingdom^d; National Mycobacterium Reference Laboratory, Public Health England, London, United Kingdom^e

Virulence factors (VFs) contribute to the emergence of new human *Mycobacterium tuberculosis* strains, are lineage dependent, and are relevant to the development of *M. tuberculosis* drugs/vaccines. VFs were sought within *M. tuberculosis* lineage 3, which has the Central Asian (CAS) spoligotype. Three isolates were selected from clusters previously identified as dominant in London, United Kingdom. Strain-associated virulence was studied in guinea pig, monocyte-derived macrophage, and lysozyme resistance assays. Whole-genome sequencing, single nucleotide polymorphism (SNP) analysis, and a literature review contributed to the identification of SNPs of interest. The animal model revealed borderline differences in strain-associated pathogenicity. *Ex vivo*, isolate C72 exhibited statistically significant differences in intracellular growth relative to C6 and C14. SNP candidates inducing lower fitness levels included 123 unique nonsynonymous SNPs, including three located in genes (*lysX*, *caeA*, and *ponA2*) previously identified as VFs in the laboratory-adapted reference strain H37Rv and shown to confer lysozyme resistance. C72 growth was most affected by lysozyme *in vitro*. A BLAST search revealed that all three SNPs of interest (C35F, P76Q, and P780R) also occurred in Tiruvallur, India, and in Uganda. Unlike C72, however, no single isolate identified through BLAST carried all three SNPs simultaneously. CAS isolates representative of three medium-sized human clusters demonstrated differential outcomes in models commonly used to estimate strain-associated virulence, supporting the idea that virulence varies within, not just across, *M. tuberculosis* lineages. Three VF SNPs of interest were identified in two additional locations worldwide, which suggested independent selection and supported a role for these SNPs in virulence. The relevance of lysozyme resistance to strain virulence remains to be established.

Human and animal tuberculosis (TB) is caused by a group of closely related bacteria (*Mycobacterium tuberculosis*, *M. canettii*, *M. africanum*, *M. microti*, *M. bovis*, *M. caprae*, *M. pinnipedii*, *M. mungi*, and *M. orygis*) collectively referred to as the *Mycobacterium tuberculosis* complex (MTBC) (1–6). *M. tuberculosis* is the main agent of TB in humans. Within *M. tuberculosis* itself, single nucleotide polymorphisms (SNPs) and large sequence polymorphisms (LSPs) are used to further classify *M. tuberculosis* into four (*sensu stricto*) major lineages, each of which is found to strongly associate with specific geographic human populations (7). While strong lineage associations with specific human populations or regions of the world are well acknowledged (8–12), the causes and relevance of this to bacterial virulence and clinical presentation are still only partly understood (13–16). It has become clear, however, that the previously unrecognized genetic diversity among *M. tuberculosis* isolates has an impact on the outcome of infection (17–19), and different approaches have been undertaken towards a better understanding of what makes a strain virulent. Some studies have attempted to tie epidemiological data to mechanistic explanations; for example, emerging Beijing strains are believed to be more virulent or associated with increased progression to disease (18, 20–22), and their glycolipids are believed to contribute to their phenotype (23–26). Outbreak strains were also found to have remarkable growth properties in animal models (27, 28) or to induce a differential protective host response (29–31). Others have focused on demonstrating that the vast genetic diversity seen

in human *M. tuberculosis* isolates is reflected in their biological properties *ex vivo* (26, 32–35) and *in vivo* (36). It is also becoming clear that the strong clonal evolution of *M. tuberculosis* (37), coupled with an apparent absence of lateral genetic exchange between lineages, is favorable for the acquisition of independent pathogenic characteristics within lineages (38). Of particular interest to the present study are studies which devoted their efforts to the identification of so-called virulence factors (VFs), especially those which combined the use of isogenic H37Rv strains with the use of animal and monocyte-derived macrophage models (39–41). In

Received 16 December 2014 Returned for modification 12 January 2015
Accepted 9 March 2015

Accepted manuscript posted online 16 March 2015

Citation Pardieu C, Casali N, Clark SO, Hooper R, Williams A, Velji P, Gonzalo X, Drobniowski F. 2015. Correlates between models of virulence for *Mycobacterium tuberculosis* among isolates of the Central Asian lineage: a case for lysozyme resistance testing? *Infect Immun* 83:2213–2223. doi:10.1128/IAI.03080-14.

Editor: S. Ehrt

Address correspondence to Claire Pardieu, c.pardieu@qmul.ac.uk, or Francis Drobniowski, f.drobniowski@qmul.ac.uk.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.03080-14>.

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doi:10.1128/IAI.03080-14

TABLE 1 Main strain characteristics

Strain	Spoligotype ^a	SNP lineage ^b	LSP lineage ^c	Characteristics ^d (isolate or laboratory-adapted strain, <i>n</i> , cluster size [by 15-locus MIRU-VNTR typing], patient's country of origin or strain virulence)	Reference
E ^e	Beijing	2	East Asian	Clinical isolate, >100, large cluster, Estonia	44
L1 ^e	LAM	4	Euro-American	Clinical isolate, >100, large cluster, United Kingdom	45
C6 ^{e,f}	CAS	3	East African-Indian	Clinical isolate, 23, medium-sized cluster, India	This study
C14 ^{e,f}	CAS	3	East African-Indian	Clinical isolate, 53, medium-sized cluster, Somalia	This study
C72 ^{e,f}	CAS	3	East African-Indian	Clinical isolate, 13, medium-sized cluster, Somalia	This study
H37Rv ^g	T	4	Euro-American	Laboratory-adapted strain, virulent	73
H37Ra ^g	T	4	Euro-American	Laboratory-adapted strain, avirulent	73
BCG ^h	NA	NA	NA	Pasteur vaccine strain, avirulent	74
C40 ^f	CAS	3	East African-Indian	Clinical isolate, 1, unique	This study

^a Isolates of the core panel were characterized by spoligotyping (46, 47). LAM, Latin American and Mediterranean; CAS, Central Asian; NA, not applicable.

^b SNP, single nucleotide polymorphism (9).

^c LSP, large sequence polymorphism (10).

^d MIRU-VNTR typing, mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing (47).

^e Core panel used in the *in vivo* model.

^f C6, C14, C40, and C72 share the CAS spoligotype and were used for genomic analysis *in silico*.

^g H37Ra and H37Rv were used as references for avirulent and virulent strains, respectively, both *ex vivo* and *in vitro*.

^h Bacillus Calmette-Guérin is an attenuated strain of *Mycobacterium bovis* and was used *ex vivo*.

the present study, we set out to identify new VFs (42) by using clinically relevant *M. tuberculosis* isolates instead of the laboratory-adapted reference strain H37Rv. Since VFs are likely to be lineage specific (25, 38, 43), we focused on three strains from an identical lineage that were isolated in a population-based study of TB in London over a period of 1 year; each isolate of interest was selected from one of the three commonest clusters of that year, according to 15-locus mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing (37). We hypothesized that these strains might perform differently in various models commonly used for the study of *M. tuberculosis* virulence as a result of slight variations in their genomes (some of which potentially encode VFs) and as suggested by their associated and differential human cluster sizes. Using isolates from ongoing outbreaks as a reference (44, 45), we first aimed to quantify the relative abilities of the strains to cause disease in an animal model. Further phenotypic and genetic markers of virulence were sought through *ex vivo* and *in silico* approaches, respectively. SNPs of interest were predicted to have an impact (or not) on protein function *in silico*, and the hypothesis that lysozyme sensitivity among Central Asian (CAS) isolates was linked to the presence of those particular SNPs was tested *in vitro*. The relevance of our findings is discussed in the broader context of strain evolution.

MATERIALS AND METHODS

Strain selection. Isolates were characterized by spoligotyping (46), which correlates with LSP analysis (10) and MIRU-VNTR typing (37). One culture from every TB patient identified (*n* = 2,261) over a 1-year period in London, United Kingdom, was subjected to a combination of 15-locus MIRU-VNTR typing and spoligotyping (47) to estimate the prevalence of circulating London strains, as well as to define their respective lineages. Isolates that could be characterized fully (*n* = 2,046) were assigned to lineages, and cluster analysis grouped 1,225 profiles into 235 clusters, each varying in size from 2 to 53 isolates. There were 1,036 profiles, so-called unique isolates, which did not fall into any cluster. The largest cluster (*n* = 53) of isolates belonged to the spoligotype family CAS (of the East African-Indian lineage), from which isolate C14 was selected. In all, CAS strains constituted 25% of the *M. tuberculosis* population, from which isolates C6 and C72 were also selected. Latin American and Mediterranean strain 10 (LAM10) L1 and an East Asian Beijing E strain were used as references for

high pathogenicity/virulence in the *in vivo* and *ex vivo* models (44, 45). Together with strains L1 and E, C6, C14, and C72 are referred to as the “core panel” herein. For *ex vivo* and *in vitro* fitness experiments, an additional CAS isolate that is unique and fully sensitive to standard TB drugs (C40) was also selected from the PHE National Mycobacterium Reference Laboratory (NMRL) database. Reference laboratory strains H37Rv and H37Ra and the *M. bovis* vaccine strain BCG (bacillus Calmette-Guérin) were used as additional benchmarks for virulent, avirulent, and attenuated strains, respectively. The main strain characteristics are listed in Table 1.

Human population characteristics. Summaries of the routine laboratory data collected by the NMRL regarding patient demographics, strain sensitivities, and sample types are shown in Tables 2 and 3. The full data can be viewed in Dataset S1 in the supplemental material.

Preparation of bacterial stocks. Bacteria were grown from early-passage stocks to mid-logarithmic phase in 7H9 Middlebrook medium (Fluka, Sigma-Aldrich) containing 0.1% Tween 80 (Sigma-Aldrich) (MTBMed) supplemented with oleic acid-albumin-dextrose-catalase (OADC; Becton, Dickinson) for 7 to 10 days at 37°C. Following two passages with a 30-fold dilution each time, bacteria were washed twice in

TABLE 2 Sample types and strain sensitivities^a

Characteristic	% of samples (% valid for χ^2 test)				<i>P</i> value
	Total (<i>n</i> = 89)	C6 (<i>n</i> = 23)	C14 (<i>n</i> = 53)	C72 (<i>n</i> = 13)	
Sample type	<i>n</i> = 79	<i>n</i> = 23	<i>n</i> = 45	<i>n</i> = 11	0.996
Sputum	47.2 (53.2)	47.8	47.2 (55.6)	46.2 (54.5)	
Fluids ^b	23.6 (26.6)	34.8	20.8 (24.4)	15.4 (18.2)	
Tissue ^c	14.6 (16.5)	13.0	15.1 (17.8)	15.4 (18.2)	
Urine	3.4 (3.8)	4.3	1.9 (2.2)	7.7 (9.1)	
Unknown culture	11.2 (0)	0	15.1 (0)	15.4 (0)	NA
Drug resistance	<i>n</i> = 80	<i>n</i> = 23	<i>n</i> = 46	<i>n</i> = 11	0.0624
Drug sensitive	74.2 (82.5)	95.7	71.7 (82.6)	46.2 (54.5)	
Isoniazid resistant	11.2 (12.5)	0	9.4 (10.9)	38.46 (45.5)	
Streptomycin resistant	4.5 (5)	4.3	5.7 (6.5)	0	
Not tested	10.1 (0)	0	13.2 (0)	15.4 (0)	NA

^a Routine reference laboratory data for the isolates were analyzed based on percentages, using the Kruskal-Wallis test (χ^2). NA, not applicable.

^b Pus, aspirates, washings, fluids, ascites, MGIT cultures, and cerebrospinal fluids.

^c Biopsy specimens, tissues, and lymph nodes.

TABLE 3 Patient characteristics^a

Characteristic	Value				P value
	Total (n = 89)	C6 (n = 23)	C14 (n = 53)	C72 (n = 13)	
Median (IQR) age (yr) ^b	n = 81 31.5 (24–44)	n = 23 38 (29–53)	n = 47 30 (22–39)	n = 11 42 (27–62)	NS
Sex (total % [% valid for χ^2 test]) ^c	n = 61	n = 16	n = 38	n = 7	
Female		30.4 (43.8)	24.5 (34.2)	15.4 (28.6)	0.9862
Male		39.1 (56.2)	47.2 (65.8)	38.5 (71.4)	
Unknown		30.5 (0)	28.3 (0)	46.1 (0)	NA
Country or region of origin (total % [% valid for χ^2 test]) ^c	n = 59	n = 18	n = 32	n = 9	
Bulgaria/Hungary	2.2 (3.4)	0 (0)	3.8 (6.3)	0 (0)	<0.0001
East Africa	33.7 (50.8)	8.7 (11.1)	49.1 (81.3)	15.4 (22.2)	
India, Pakistan, or Nepal	24.7 (37.3)	65.2 (83.3)	0 (0)	53.8 (77.8)	NA
UK	5.6 (8.5)	4.3 (5.5)	7.5 (12.5)	0 (0)	
Unknown	33.7 (0)	21.7 (0)	39.6 (0)	30.8 (0)	

^a Routine reference laboratory data on the patients. NA, not applicable.^b Medians and 1st and 3rd interquartile ranges (IQRs) for age were generated by using the bootstrap theory. NS, nonsignificant.^c Data were analyzed based on percentages, using the Kruskal-Wallis test (χ^2).

Dulbecco's phosphate-buffered saline (DPBS)–0.05% Tween 80, and the optical density at 650 nm (OD₆₅₀) was measured. OD₆₅₀ measurements at the time of aliquot preparation varied between 0.4 and 0.9 (early to late log phase). All strains were suspended in MTBMed-10% glycerol before 20- μ m filtration (Millipore) to remove visible clumps. The OD₆₅₀ was adjusted to around 0.1 (0.08 to 0.12), and aliquots were stored at -80°C .

In vivo infection model. *In vivo* experimental work was conducted at PHE-Porton Down according to UK Home Office legislation for animal experimentation and was approved by the local ethical committee. Mycobacterial aerosols were generated by a three-jet Collison nebulizer with a contained Henderson apparatus (48) as previously described (49). Isolates were randomly assigned to guinea pigs; groups of eight Dunkin Hartley female guinea pigs (Harlan Laboratories, Inc.) were infected with each isolate. The starting bacterial suspension concentration was 10^4 CFU/ml, to deliver an estimated retained dose of 10 CFU/lung (50, 51). At necropsy (8 weeks postchallenge), animals and lungs were weighed before lung sections were fixed, embedded, and sectioned for histopathology analysis. Histopathology scores were attributed based on granuloma disease severity, i.e., the degree of consolidation, caseation/necrosis, and calcification (50). Spleen bacterial loads were determined by the CFU method.

In vitro lysozyme sensitivity assay. Bacterial stocks were thawed, serially diluted in triplicate, and plated (10 μ l) on 7H10 agar plates prepared per the manufacturer's recommendations, but with a 20-mg/ml stock solution of chicken lysozyme (Sigma-Aldrich) added to the desired final concentration. Colonies were counted after 3 to 5 weeks at 37°C .

Ex vivo MDM infection model. Buffy coats were isolated from the reconstituted whole blood of healthy donors (NHS Blood Bank, Brentwood, United Kingdom) by density gradient centrifugation using Lymphoprep (Axis-Shield) and were washed thrice in no- Ca^{2+} /Mg²⁺ DPBS (Life Technologies). Monocytes positively selected with CD14 microbeads (Miltenyi Biotec) were differentiated for up to 7 days in RPMI medium (Sigma-Aldrich) supplemented with 10% human A/B serum (PAA Laboratories, GE Healthcare) and 5 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gibco, Life Technologies), detached in the presence of cold DPBS (no Ca^{2+} /Mg²⁺), and seeded at 5×10^4 cells/well in 96-well plates before infection. Monocyte-derived macrophage (MDMs) were infected at a multiplicity of infection (MOI) of 10 (5×10^5 *rpoB* copies/well). At 4 h (day 0 [D0]) postinfection, extensive washes were performed in RPMI medium, MDMs were lysed with 200 μ l of distilled and deionized water for 10 min and pipetted up and down 10 times, and lysates were analyzed by quanti-

tative PCR (qPCR). The effective MOI, as determined at D0, was close to 1 bacillus (*sigA* or *rpoB* copy)/MDM.

qPCR assay. Samples were heat inactivated at 80°C for 45 min. Bacilli were disrupted by beating twice for 45 s each with acid-washed beads ($<106 \mu\text{m}$; Sigma-Aldrich) in a RiboLyser apparatus (Hybaid). Supernatants (4 μ l) of centrifuged ($14,000 \times g$) samples were used as templates in a final volume of 20 μ l. Bacterial stocks and samples were titrated using the primer pair *rpoB*-F (GCCGATCAGACCGATGTTG) and *rpoB*-R (CCGCGATCAAGGAGTTCTTC) or *sigA*-F (CCGATGACGACGAGGAGATC) and *sigA*-R (GGCCTCCGACTCGTCTTCA) at 300 nM (each primer) in a SYBR green I assay (PerfeCTa SYBR green SuperMix; Quanta Biosciences).

Lysates of *M. tuberculosis*-infected MDMs were assayed using a duplex qPCR assay; primers *sigA*-F and *sigA*-R were used together with *hTERT*-F (GGCACACGTGGCTTTTCG), *hTERT*-R (GGTGAACCTCGTAAGTTATGCAAACTG), and probes for *sigA* (6-carboxyfluorescein [FAM]-AAGGACAAGGCCTCCGGTGATTTTCG-BHQ1) and the reference human gene *hTERT* (telomerase reverse transcriptase gene) (HEX-TCAGGACGTCGAGTGGACACGGTG-BHQ1), at a 100 nM final concentration of each, using PerfeCTa SuperMix (Quanta Biosciences). Reactions were carried out in triplicate (aliquots) or duplicate (*M. tuberculosis*-infected MDM technical repeats, i.e., quadruplicates for each condition tested) on an iQ5 PCR detection system (Bio-Rad) per the manufacturer's instructions, with the following cycling conditions: 35 cycles of 95°C for 10 s and 66°C for 45 s (*sigA*) or 95°C for 10 s and 65°C for 35 s (*rpoB*). For all qPCR assays, a standard curve was derived from the threshold cycle (C_T) values obtained for reactions performed on a 10-fold serially diluted *M. tuberculosis* preparation, using the *sigA* or *rpoB* primer pair. For duplex assays, a standard curve for human genome copy quantification was simultaneously obtained using 10-fold serially diluted human genomic DNA (Roche).

CFU determination method. Tenfold serial dilutions of bacterial stocks (20 μ l) performed in triplicate in 7H9 Middlebrook medium were plated (10 μ l) on 7H10 Middlebrook agar plates (prepared per the manufacturer's instructions). Colonies were grown for 3 weeks at 37°C , and dilutions first showing distinct colonies were used for enumeration. For spleen CFU, enumeration was performed on 7H11 Middlebrook agar plates, using 100- μ l aliquots of serially diluted spleen homogenates (49).

Genomic analysis. Paired-end multiplex libraries were sequenced on an Illumina HiSeq platform. Sequence reads were mapped to a corrected version of the H37Rv reference genome (52) by using SMALT (www.sanger.ac.uk/resources/software/smalt). High-quality SNPs were identi-

fied and variant positions used to construct a maximum likelihood tree (53). SNPs located in repetitive regions were excluded from the analysis. Artemis (release 15.0.0) was used for genome visualization and comparison. The SIFT (54) and PROVEAN (55) algorithms were used to predict the effect that nonsynonymous SNPs (nsSNPs) of interest might have on protein function. Several SIFT functions were used, including “blink,” “sequence,” and “related sequences,” and we selected the final scores achieving good levels of confidence as well as falling within the recommended median conservation (of sequences) range (2.75 to 3.25).

Data and statistical analyses. (i) *In vivo* model. Analysis of variance was used to investigate guinea pig weight-change differences between isolates over time, i.e., an interaction between isolate and time was sought. Guinea pigs (all weighing within 50 g of each other at purchase) were randomized to receive different isolates. Weights at day 51 were also compared between isolates and were analyzed using covariance analysis, with adjustment for baseline weights. Other measures of virulence were compared between strains by using the nonparametric Kruskal-Wallis test.

(ii) *Ex vivo* model. Bacillus/MDM numbers were determined for lysates by using duplex qPCR for *sigA* and *hTERT* and calculating the ratios of *sigA* to *hTERT* copies. Fitness was estimated as the ratio of values at D5 relative to D0 (D5/D0). An analysis of variance of the (natural) log-transformed D5/D0 ratios relative to that for H37Ra was used to investigate differences between isolates, with adjustment for a random effect of the batch of stocks used and for fixed effects of the strain and donor. Ratios are presented following their exponentiation back to the linear scale.

(iii) *In vitro* model. CFU values enumerated from agar plates containing 2 or 4 mg/ml of lysozyme, or none, were log transformed before analysis. In cases where no colonies could be recorded, a value of 0.05 was assumed. Analysis of variance was used to investigate differences between strains in the linear effect of lysozyme on CFU (i.e., an interaction between strain and lysozyme was sought), with adjustment for systematic differences between replicates ($n = 2$).

RESULTS

***M. tuberculosis* strain selection hypothesis.** *M. tuberculosis* strains used in the present study were selected as described in Materials and Methods. Their main characteristics are listed in Table 1. Three CAS isolates of lineage 3 (37) were selected from clusters of similar yet nonidentical sizes based on 15-locus MIRU-VNTR analysis. We hypothesized that subtle epidemiological differences should translate into differential readouts in *ex vivo* and *in vivo* models of virulence (22, 27) and that identification of corresponding VFs should be facilitated *in silico* through the elimination of lineage-defining regions of difference (RDs), SNPs, or LSPs. Of the 2,261 isolates identified in London in the year examined, the isolates of interest represented 0.57% (C72; $n = 13$), 1.02% (C6; $n = 23$), and 2.34% (C14; $n = 53$) of the total population, with 95% confidence intervals of 0.32 to 1%, 0.66 to 1.55%, and 1.77 to 3.07%, respectively, suggesting a higher prevalence of C14 than of C72 or C6. C40 was picked at random from isolates in the NMRL database that were both unique and of the CAS spoligotype.

Human and strain population characteristics. Strain characteristics, including *in vitro* drug sensitivities and the type of sample from which each isolate originated, were analyzed to provide insights into strain-associated virulence in humans (Table 2). Interestingly, although sample types did not show significant differences between strains ($P = 0.996$), suggesting an equal or lack of ability to disseminate intrahost (Table 2; see Fig. S2C in the supplemental material), there was borderline significance between strains regarding their resistance to isoniazid and streptomycin ($P = 0.0624$). Patient characteristics are shown in Table 3. Neither age (medians and interquartile ranges) nor gender distributions

significantly differed across populations; however, the population from which C14 was isolated was found to originate mainly from East Africa, while the human populations infected with C6 and C72 came mainly from India, Pakistan, or Nepal ($P < 0.001$).

***In vivo* fitness model: *M. tuberculosis* infection of guinea pigs.** The virulence of the core panel was determined in guinea pigs (Fig. 1). Changes in body weight over the course of infection did not show significant differences between isolates ($P = 0.15$), and there was no evidence of differences at day 51 ($P = 0.45$), when animals were last weighed together (Fig. 1A). In organs taken at necropsy, no evidence of differences in pathogenicity scores could be established between C6, C14, and C72 ($P = 0.70$) (Fig. 1B), but there was a trend toward differential virulence according to spleen CFU data ($P = 0.076$) (Fig. 1C) and the ratios of lung to body weights ($P = 0.0502$) (Fig. 1D).

***Ex vivo* fitness model: intracellular growth of *M. tuberculosis* in MDMs.** MDMs from six healthy donors were used for *ex vivo* infections with the core panel, virulent/avirulent strains H37Rv/H37Ra, BCG, and an additional clinical isolate, C40 (Table 1). We measured the change in *M. tuberculosis* copy number per cell over 5 days by using duplex qPCR (see Materials and Methods) and used that as a measure of *M. tuberculosis* fitness. Fitness was analyzed relative to that of H37Ra (Fig. 2).

Remarkably, there was strong evidence of differences between strains in their fitness levels *ex vivo*. All clinical isolates differed significantly from the avirulent reference strains H37Ra and BCG ($P < 0.001$). Importantly, among the three medium-sized clusters of CAS isolates of the core panel, C72's estimated fitness was found to be the lowest ($P < 0.001$) and was as low as that of C40, which was not associated with any cluster and therefore unique.

Genomic analysis. Comparative genomic analysis was performed with the aim of identifying candidate genomic variations and SNPs unique to C72 to explain its differential fitness level in MDMs (Fig. 2). We restricted our *in silico* analysis to all four CAS isolates (C6, C14, C72, and C40) to eliminate LSPs, RDs, and SNPs that define lineages and to reduce the impact that the genetic background might have on VF-associated (25, 38, 43) phenotypes. We first searched for the presence/absence of RD149 (*Rv1573-Rv1584c*), as its absence was previously associated with reduced intracellular growth of CAS strains in THP-1 cells (56). Interestingly, the RD149 deletion was uniquely identified in C72 (see Fig. S3 in the supplemental material), not in any of the other CAS strains analyzed. Additionally, 221 SNPs unique to C72 were identified. Among those, 123 are nonsynonymous (ns), 69 synonymous (s), and 28 intergenic, and 1 lies in an RNA-encoding region (see Table S4). We searched for reports in the literature of corresponding genes whose deletion was determined to have a significant impact on *M. tuberculosis* fitness *ex vivo* or *in vivo*, particularly among the 194 major candidate genes established as crucial for *in vivo* survival and dissemination in mice by Sasseti and Rubin (40) and the 126 candidates identified as promoting survival in macrophages by Rengarajan et al. (41). As a result, nine unique SNPs were identified as candidates for C72's observed differential phenotype *ex vivo* (Table 4). Of those, three were thought to be of particular interest due to their nonsynonymous nature and because the corresponding genes are commonly associated with the natural resistance of *M. tuberculosis* to lysozyme (57–59), in addition to being labeled VFs (42). Using the SIFT (54) and PROTEAN (55) algorithms, the P76Q substitution was predicted to be likely to affect protein function. Thus, we hypothesized that C72 would

FIG 1 *In vivo* phenotypes of the core panel. Five groups of eight female Dunkin Hartley guinea pigs were infected via the aerosol route as described in Materials and Methods. (A) Mean weights of animals infected with isolates E (yellow), L1 (pink), C6 (blue), C14 (green), and C72 (red) were recorded over 8 weeks. An analysis of variance of weight change over time revealed no significant differences between isolates ($P = 0.15$) or any evidence of differences in weight at day 51 ($P = 0.45$). (B to D) Animals were culled at 8 weeks postchallenge and virulence further assessed. (B) Lung pathogenicity scores were determined by a trained histopathologist and were based on relative extents of consolidation/necrosis/caseation and calcification. (C) *M. tuberculosis* that had disseminated from the infected lungs to the spleen at the time of sacrifice was quantified by the CFU method. (D) Lungs and animals were weighed at necropsy and corresponding ratios calculated. No significant differences were found among the CAS isolates of the core panel in their lung pathogenicity scores ($\chi^2 = 0.72$; $\text{df} = 2$; $P = 0.70$). There were only borderline differences in weight ratios between C6, C14, and C72 ($\chi^2 = 5.98$; $\text{df} = 2$; $P = 0.0502$) and in spleen titers ($\chi^2 = 5.17$; $\text{df} = 2$; $P = 0.076$).

demonstrate differential sensitivity to lysozyme compared with the other CAS strains included in this study and that this property could be used as a surrogate measure of the impacts of mutations on other functions associated with *lysX*, *ponA2* (58, 60), and *caeA* (57, 60, 61), in particular.

***In vitro* sensitivity to lysozyme.** The core panel, C40, and the reference strains (H37Ra/H37Rv) were next tested for resistance to increasing concentrations of lysozyme *in vitro*, with the underlying hypothesis that C72 would have differential sensitivity to it as a consequence of uniquely bearing nsSNPs in Rv1640c (*lysX*), Rv2224c (*caeA*), and Rv3682 (*ponA2*). Interestingly, all strains were found to be sensitive to lysozyme to some extent ($P < 0.05$) (Fig. 3), and strong variations between strains could be observed in the degree of their susceptibility to it ($P < 0.001$). Significant differences between C6, C14, and C72 were observed, with C6 and C14 showing less susceptibility to lysozyme than C72 did (Fig. 3) ($P < 0.001$), correlating with the presence of the nsSNPs of interest within the core panel. However, C72 did not show statistically significant differences in sensitivity compared to the unique CAS isolate C40, suggesting that additional determinants of lysozyme resistance are likely to lie elsewhere in the *M. tuberculosis* genome.

Estimated frequencies of nsSNPs in *lysX*, *ponA2*, and *caeA* worldwide. Because the various models of virulence we used in this study did not converge toward a strong consensus for any single CAS strain of interest being more (or less) virulent than the other two, and because the data that were available to us regarding the human population characteristics from which they were isolated (Table 3) were also limited, we could not formally infer that

FIG 2 Among the core panel CAS isolates, C72 had the lowest fitness level *ex vivo*. MDMs from four donors were infected in duplicate ($n = 2$) or triplicate ($n = 3$) with clinical isolates of interest C6, C14, C72, E (44), and L1 (45) alongside laboratory-adapted reference strains H37Ra (Ra) and H37Rv (Rv) (73) and vaccine strain BCG (74). MDMs from an additional two donors were also infected, in triplicate, with the unique isolate C40, alongside the core panel, H37Ra, H37Rv, and BCG. Heat-killed (HK) samples were used as controls for an absence of *M. tuberculosis* (MTB) growth. The number of bacilli per MDM was estimated by duplex qPCR for the bacterial *sigA* and human *hTERT* genes, using cell lysates recovered at 4 h (D0) and 5 days (D5) postinfection. The ratio of *M. tuberculosis* copies/MDM at D5 over D0 (D5/D0) was analyzed relative to the same ratio for H37Ra, using an analysis of variance. Data are presented with 95% confidence intervals and demonstrate significant differences in fitness between strains ($P < 0.001$). In particular, among the CAS isolates of the core panel, C72 exhibited the lowest estimated fitness level ($P < 0.001$).

TABLE 4 SNPs unique to C72 and corresponding gene deletions^a

Rv no.	Gene name	SNP	Type of SNP	Effect of deletion in BMDMs ^b			Effect of deletion in mice ^c		In silico prediction		
				No IFN- γ	IFN- γ (pre)	IFN- γ (post)	Wk postinfection	P value	SIFT score ^d	PROVEAN score ^e	Effect ^f
Rv0020c	<i>fhaA</i>	R480R	s	—	—	NS	NS	NA	NA	NA	NA
Rv0169	<i>mce1A</i>	K185K	s	—	—	—	2	<0.02	NA	NA	NA
Rv0426c	Rv0426c	G47G	s	—	NS	—	NS	NA	NA	NA	NA
Rv0994	<i>moeA1</i>	A273A	s	+	NS	NS	NS	NA	NA	NA	NA
Rv1332	Rv1332	T76T	s	NS	—	NS	2	<0.02	NA	NA	NA
Rv3563	<i>fadE32</i>	Q202Q	s	—	—	NS	1, 8	<0.005	NA	NA	NA
Rv1640c	<i>lysX</i>	C35F	ns	—	—	—	4	<0.005	0.32	−0.085	N
Rv2224c	<i>caeA</i>	P76Q	ns	—	—	—	2, 4	<0.005	0.00	−7.987	D
Rv3682	<i>ponA2</i>	P780R	ns	NS	—	—	NS	NA	0.22	−1.466	N

^a The genes corresponding to SNPs of interest (i.e., unique to C72 but not seen in C6, C14, or C40) were investigated in the literature. Genes highlighted in gray uniquely carry nsSNPs (as opposed to sSNPs) in C72. NA, not applicable; NS, nonsignificant.

^b Effects of specific gene deletions in H37Rv were analyzed in bone marrow-derived macrophages (BMDMs). “IFN- γ (pre),” IFN- γ was added to the macrophages before infection with the deleted strain; “IFN- γ (post),” IFN- γ was added to the macrophages after infection with the deleted strain. The “+” and “−” signs correspond to the respective positive and negative effects that the mutations were considered to have (41).

^c Number of weeks postchallenge at which the effect of the specific gene deletion in H37Rv was analyzed as deleterious, together with the corresponding P value (40).

^d Score predicted in silico using the Sorting Intolerant From Tolerant (SIFT) algorithm (54).

^e Score predicted in silico using the Protein Variation Effect Analyser (PROVEAN) algorithm (55).

^f N, neutral; D, deleterious.

the observed differences in cluster-associated sizes for C6, C14, and C72 in London that year were actually reflective of clinically significant differences in their strain-associated virulence. We therefore searched the NCBI databases, using the BLAST tool, for the presence of our three nsSNPs of interest, in *lysX* (C35F), *caeA* (P76Q), and *ponA2* (P780R), in all other deposited *M. tuberculosis* genomic sequences to help to characterize their frequencies and significance worldwide (Table 5). Remarkably, a total of at least 85

strains carrying any of the three mutations of interest could be identified worldwide, including in India, Uganda, Iran, South Africa, and Sweden (see Dataset S5 in the supplemental material). However, the occurrence of isolates carrying any of the three mutations of interest and coexisting in a given location could be observed only in Tiruvallur, India, and in Uganda; isolates for which (i) all three genes of interest could be retrieved and (ii) the date and exact location (i.e., a city name) were documented are presented here (Table 5). In Tiruvallur, 32 isolates (21.2%) retrieved from sequences deposited between 1905 and 2003 were found to carry the nsSNP P780R (*ponA2*); only five strains carried the other two nsSNPs of interest (C35F and P76Q) together, and just one (isolate M2440) carried the C35F SNP (*caeA*) on its own. Interestingly, no isolate bearing all three mutations simultaneously, as observed for C72, was identified.

DISCUSSION

M. tuberculosis isolates found in epidemiologically defined clusters are defined as successful because they transmit and cause TB in more human hosts, in the same population, than the case for other isolates. It is also a generally accepted idea that overall strain success depends on a combination of strain virulence traits and host genetic factors (8, 14, 16, 62, 63). The contribution of mycobacterial VFs to establishing disease (TB) or causing pathogenicity, however, was recently reemphasized (42, 64); as VFs are likely to be lineage specific (23–25, 38, 43), their existence might have far-reaching impacts on the efficacy of new TB vaccines or drugs (65).

In this study, our approach was to identify naturally successful strains of *M. tuberculosis* from the population of London, United Kingdom, and to use the knowledge from previous *in vivo* and *ex vivo* studies carried out with isogenic strains (39–41) to develop hypotheses on and confirm/discover VFs relevant to their biology. We focused on closely related isolates from the CAS spoligotype family to minimize the potential impact that genetic background variability has on VF-associated phenotypes (25, 43). Based on 15-locus MIRU-VNTR typing, each isolate of interest was associ-

FIG 3 CAS isolate C72 is the most sensitive of the core panel isolates to lysozyme *in vitro*. Aliquots of isolates from the core panel and reference strains H37Rv (Rv) and H37Ra (Ra) (73) were thawed and serially diluted in triplicate before plating (10 μ l) onto 7H10 plates containing OADC only or OADC with 2 or 4 mg/ml of chicken lysozyme (final concentration). The experiment was replicated using different batches of plates ($n = 2$) and, in the case of C72 and C14, different bacterial stocks ($n = 2$) to eliminate potential batch effects. E (44), L1 (45), C6, C14, C72, and C40 were tested twice and Rv and Ra only once. Colonies were counted after 4 to 5 weeks of growth at 37°C, and the values were log transformed. When CFU were undetectable, even at the lowest dilution, a value of 0.05 was assumed. The relative reduction (fold change) for each strain is shown with the 95% confidence interval. An analysis of variance on the estimated fold change in CFU per mg/ml of lysozyme demonstrated lysozyme sensitivity for all strains ($P < 0.001$) and showed C72 to be the most sensitive among the CAS isolates of the core panel ($P < 0.001$).

TABLE 5 Isolates from Tiruvallur, India, carrying nsSNPs of interest C35F, P76Q, and P780R^a

Isolate(s)	Yr of isolation	SNP			<i>n</i>	% of isolates with SNP pattern	
		Rv1640c	Rv2224c	Rv3682		Overall	That year
M1030, M2352	1905	WT	WT	WT	2	1.3	100
M1017	2001	WT	WT	P780R	1	0.7	5.6
WT in 2001	2001	WT	WT	WT	17	11.3	94.4
M1704, M1780, M1787, M1913, M1955, M2248, M2191, M2324, M2323, M2249, M2402, M2376, H2101, M2828, M2193, M1759	2002	WT	WT	P780R	16	10.6	26.2
WT in 2002	2002	WT	WT	WT	45	29.8	73.8
M2479, M2508, M1270, M1274, M1292, M1294, M1309, M1374, M1393, M1459, M1488, M1545, M1559, M1638, M1702	2003	WT	WT	P780R	15	9.9	21.4
M2440	2003	C35F	WT	WT	1	0.7	1.4
M1762, M2006, M2007, M2277, M2276	2003	C35F	P76Q	WT	5	3.3	7.1
WT in 2003	2003	WT	WT	WT	49	32.5	70
Singly mutated isolates ^b	1905–2003	C35F or P780R			33	21.9	NA
Doubly mutated isolates ^c	1905–2003	C35F and P76Q			5	3.3	NA
Total mutated isolates ^d	1905–2003	P780R, C35F, or P76Q			38	25.2	NA
Total WT isolates	1905–2003	WT only			113	74.8	NA
Total	1905–2003	All			151	100.0	NA

^a The NCBI databases were searched for any of the three nsSNPs of interest occurring worldwide by using BLAST. Only the isolates for which all three sequences for the genes of interest (*lysX*, *caeA*, and *ponA2*) could be retrieved were considered for further analysis. Of a total of 85 isolates identified as carrying any of the three SNPs of interest in a given country, 38 occurred in a specific city, Tiruvallur, India. In Tiruvallur, a total of 151 isolates (WT and mutated) were retrieved using BLAST. WT, wild type; NA, not applicable.

^b Of the total isolates retrieved, 33 (21.9%) carried single mutations, with the vast majority carrying the P780R mutation (32/33 isolates).

^c Of the total isolates retrieved, only 5 (3.3%) carried two mutations, C35F and P76Q, simultaneously. No isolates carrying all three mutations at once were retrieved.

^d Overall, in Tiruvallur, a quarter (25.2%) of the isolates analyzed and identified between 1905 and 2003 carried at least one of the nsSNPs of interest.

ated with a human cluster, with cluster sizes varying from 13 (C72) to 53 (C14) individuals, and we initially reasoned that this should be reflected in some way in both the *in vivo* and *ex vivo* models we used to assess the relative levels of virulence. Interestingly, in guinea pigs, there was an indication that isolate C72 was less virulent than either C6 or C14, although not at the 5% level of confidence. Spleen bacterial loads (a measure of dissemination) were lower in animals infected with C72 than in those infected with C14 or C6 at the 10% level of confidence ($P = 0.0760$). Similarly, lung/body weight ratios (a measure of general disease burden) indicated, at the 10% confidence level, that guinea pigs infected with C72 or C14 were less ill than those infected with C6 (Fig. 1D) ($P = 0.0502$). There was, however, an overall indication of similar pathogenicities between isolates (Fig. 1A to C) ($P > 0.050$) in this model. This raised the possibility that subtle genetic differences distinguishing C6, C14, and C72 might not affect their pathogenicity *in vivo* or that the (15-locus MIRU typing-based) cluster-associated sizes for C6, C14, and C72 were not related to strain-associated characteristics but to differences in the populations from which they were isolated. It was also possible that the highly susceptible host, that is, the guinea pig, was not able to clearly indicate subtle genetic differences. However, in this study, extensive measurements of dissemination (including in the heart and the adrenal glands) were not performed yet the extent of extrapulmonary lesion necrosis was shown to be a better predictor of virulence than the number of viable bacilli (28). Small effects are also less likely to be detected in studies with small numbers, such as ours (66).

In sharp contrast to the *in vivo* model, however, the *ex vivo* model using human MDMs showed significant differences among

the isolates of the core panel in their fitness *ex vivo* ($P < 0.001$), supporting the idea that differences in virulence between those strains did exist, as bacterial growth in macrophages is equally used and validated as a correlate of strain-associated virulence.

Since previous studies had linked a specific deletion (RD149) in CAS strains with less replication in THP-1 cells (56), we specifically scanned this region of the genome for isolates C6, C14, C72, and C40. Remarkably, only C72 harbored such a deletion, supporting the idea that this region is important for intracellular replication in CAS strains.

Our genomic analysis also revealed 221 SNPs unique to C72, among which 9 were located in genes previously reported to significantly affect pathogenicity and dissemination in mice (41, 57, 58) and guinea pigs (58) or survival in macrophages (41) for isogenic strains of H37Rv. Three of those SNPs were nonsynonymous (nsSNPs) and were considered candidates for contributing to C72's differential behavior *ex vivo* because they were located in genes (*lysX*, *caeA*, and *ponA2*) that, in addition to already being identified as VFs (42), had also been demonstrated to affect the natural resistance of *M. tuberculosis* to acid (61), antibiotics (58, 60, 61), and lysozyme (57, 58, 60), a property that we further assessed. Interestingly, one of the three nsSNPs, P76Q (*caeA*), was predicted to affect protein function *in silico*. Consistent with the trends observed in the guinea pig model and the data on MDMs, C72 was found to be the most sensitive to lysozyme *in vitro*, although statistical significance was not reached between C72 and C40, suggesting that there are additional determinants of lysozyme sensitivity in CAS strains. Nevertheless, our data support the results of previous studies demonstrating lysozyme sensitivity for H37Rv *in vitro* (57, 58, 60) and for *M. smegmatis* *ex vivo* (67).

We now also demonstrate a broad range of lysozyme sensitivities in *M. tuberculosis* isolates across different lineages ($P < 0.001$). Interestingly, *Mycobacterium* sensitivity to lysozyme was first reported in 1983, for *M. leprae* (68), but the identity of genes conferring such resistance was not revealed until recently (39–41, 57). Since lysozyme is the most abundant antimicrobial peptide in respiratory tract secretions and its local levels can increase about 10-fold during inflammation (69), it remains to be addressed whether and which additional *M. tuberculosis* genes are involved in such resistance and if they represent realistic drug targets. Of particular relevance to our findings will be the results of two current phase IV clinical trials (ClinicalTrials.gov identifiers NCT01645800 and NCT01715493) looking at the beneficial effect of lysozyme treatment (90-mg capsule, 3 times daily for 4 to 52 weeks) on exacerbation of chronic obstructive pulmonary disease (COPD), as there is interest in the identification of links between COPD and TB. Lysozyme supplementation may offer a new means of prevention of TB in COPD patients or for those individuals whose lysozyme levels are decreased, such as in response to environment pollutants (70–72).

To further establish the clinical relevance (to humans) of differences that were measured among our three strains of interest in the *ex vivo* model *in silico* and *in vitro*, we also investigated the characteristics of the human population from which each was isolated. The striking difference between all three populations was in their origins. While C6 and C72 were isolated from a population predominantly originating from India, Pakistan, and Nepal, C14's associated human population was predominantly from East Africa.

We also searched the NCBI databases in the hope of discovering a situation similar to that observed in London, i.e., where strains with the mutations seen in C72 (C35F, P76Q, and P780R) were underrepresented compared to those without the mutations, but in a setting where both the human population and the living conditions would be different from those of London to help differentiate strain-specific from human-specific factors. Through a BLAST search, we identified Tiruvallur, India, as well as Uganda, as two independent locations where all three mutations could also be detected. Of the 151 isolates originating from Tiruvallur, 32 carried the P780R mutation, while only 1 carried the C35F mutation, on its own, and 5 bore the C35F and P76Q mutations. None was identified as carrying all three mutations simultaneously, as observed in the isolate of interest C72. It is therefore tempting to suggest a mutually exclusive relationship between the coexistence of the C35F and P76Q mutations on the one hand and the P780R mutation on the other. Larger numbers of isolates would need to be analyzed to support this hypothesis; however, no data could be retrieved for the year 2004 or beyond for this specific location (Tiruvallur, India). Interestingly, the P780R mutation on its own could be identified in isolates from various other locations, including Iran, South Africa, and Sweden, suggesting an evolutionary advantage in carrying this specific nsSNP. It is also interesting that the C35F and P76Q mutations could not be retrieved in strains before 2003, while the P780R mutation was observed as early as 2001, and at an even higher frequency from 2002 on, in Tiruvallur. Together with the data demonstrating that the individuals infected with the C72-associated cluster were originally from India, Pakistan, or Nepal, the presence of the mutations in the *ponA2*, *lysX*, and *caeA* genes found specifically in Tiruvallur, India, strongly suggests that C72 originated there and was brought to the

United Kingdom in or after 2003. Meanwhile, the exact time and location of the emergence of C72 remain unknown, but it likely arose from an Indian isolate originally from Tiruvallur that carried both the C35F and P76Q mutations or the P780R mutation on its own.

Overall, for the strains we were interested in, growth measurement in MDMs appeared to be more sensitive than the guinea pig model for detecting *M. tuberculosis*-associated virulence factors that were small yet potentially of relevance to clinical disease in humans. Our data are insufficient, however, to suggest that qPCR measurements *ex vivo* are better virulence surrogate markers than pathogenicity scores or CFU data for guinea pigs. Nevertheless, we do suggest that while the RD149 deletion in C72 might have an impact on fitness in human macrophages (56), mutations found in *lysX*, *ponA2*, or *caeA* might confer an evolutionary advantage to balance out the lower replication capacity in macrophages. Another observation supporting the idea that no single model can capture all there is to know about *M. tuberculosis* strain virulence was the finding that a unique (C40) isolate demonstrated fitness levels similar to those of a clinical isolate from a large human cluster (C72). This indicated that although fitness levels *ex vivo* can be discriminatory between strains that are highly related to each other or from the same lineage (our study), they might not always be directly related to success in the human population or to their associated cluster size.

Taken together, our data suggest that while the mutations identified in *ponA2*, *lysX*, and *caeA* are likely to affect the strain's resistance to lysozyme, they might also positively affect the other (and maybe as yet unknown) functions of the very same genes. The P780R mutation, in particular, might confer an evolutionary selective advantage.

ACKNOWLEDGMENTS

This work was supported by the European Union Framework Programme 7 (TB-EUROGEN grant 201483 and TB PAN-NET grant 223681), Barts and The London Charity (grant 807/1619), and internal funding (PHE-Porton Down), and sequencing was funded by the Wellcome Trust through core funding to the Sanger Institute (grant 076964).

The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

We thank David van Bockel for his contribution to the qPCR assay design and Vladyslav Nikolayevsky, Madeline Stone, and Timothy Brown for their help in identifying isolate spoligotypes, as well as the PHE for providing access to the NMRL database and United Kingdom isolates. Finally, we thank Jane Deayton for helpful discussions.

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