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Original Article

Performance of a highly successful outbreak strain of *Mycobacterium tuberculosis* in a multifaceted approach to bacterial fitness assessment

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ABSTRACT

Determining bacterial fitness represents a major challenge and no single parameter can accurately predict the ability of a certain pathogen to succeed. The M strain of *Mycobacterium tuberculosis* managed to spread and establish in the community and caused the largest multidrug-resistant tuberculosis outbreak in Latin America. We have previously shown that the M strain can manipulate the host immune response, but we still have no direct evidence, other than epidemiology, that can account for the enhanced fitness of the M strain. Our objective was to further characterize the performance of the outbreak strain M in different fitness assays. Two main aspects were evaluated: (1) molecular characterization of selected isolates from the M outbreak and related strains and (2) comparative fitness and in vivo performance of representative M strain isolates vs. the non-prosperous M strain variant 410. Our approach confirmed the multifaceted nature of fitness. Altogether, we conclude that the epidemiologically abortive strain 410 was vulnerable to drug-driven pressure, a weak competitor, and a stronger inductor of protective response in vivo. Conversely, the isolate 6548, representative of the M outbreak peak, had a growth disadvantage but performed very well in competition and induced lung damage at advanced stages in spite of reaching relatively low CFU counts. Integration of these observations supports the idea that the M strain managed to find a unique path to success.

1. Introduction

Although major advances have been made in the understanding of bacterial fitness, there is still no consensus about how to predict the ability of a certain pathogen to succeed (Didelot et al., 2016). Fitness has a multifaceted nature and no single parameter can give the whole picture. Assays usually include axenic growth, resistance to stressors, competition assays, as well as in vitro and in vivo infection models (Pope et al., 2010; Wiser and Lenski, 2015). The intricate nature of host-pathogen interactions in tuberculosis further complicates the definition of fitness for its etiological agent. Indeed, within the spectrum of possible outcomes of the infection, both extremes, namely

Abbreviations: CFU, colony forming units; DTH, delayed-type hypersensitivity; EMB, ethambutol; INH, isoniazid; IS*6110*-RFLP, restriction fragment length polymorphism based on insertion sequence *6110*; KAN, kanamycin; MDM, human monocyte-derived macrophages; MDR, multidrug-resistant; MIC, minimal inhibitory concentration; MOI, multiplicity of infection; Mono-R, mono drug-resistant; OFX, ofloxacin; Pan-S, pansusceptible to drugs; pre-XDR, pre-extensively drug-resistant; PZA, pyrazinamide; RIF, rifampicin; SIT, spoligotype international type; STR, streptomycin; VNTR-MIRU, variable number of tandem repeats-Mycobacteria interspersed repetitive units; XDR, extensively-drug resistant

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Table 1
Characteristics of the selected strains.

Isolate n°/year	Clade	SIT	Strain	RFLP *IS6110*	VNTR-MIRU 15 loci	HIV status
Prototype pre-XDR/XDR M strain isolates						
6548/98	H2	2	M		254533233433537	+
12838/05	H2	2	M		254533233433537	−
15526/07	H2	2	M		254533233433**4**37	+
Non-prosperous MDR M-related strain						
410/95	H2	2	M variant	↑	254**6**33233433537	−
Non MDR M-related strains						
12000/04	H2	2	M		25**24**33233433537	−
6145/98	H2	2	M-like		254533**1**33433537	−
14674/06	H2	2	M-like		254533**1**33433**6**3**6**	−

Abbreviations: SIT: spoligotype international type; IS*6110* RFLP: restriction fragment length polymorphism based on insertion sequence *6110*; VNTR-MIRU: variable number of tandem repeats-Mycobacteria interspersed repetitive units; XDR: extensively drug-resistant; MDR: multidrug-resistance. Arrow indicates a duplicated IS*6110* band in 410 strain. MIRU-VNTR loci marked in bold are those that differ from the prototype M strain. Some data regarding isolates 6548 and 410 were previously published in Yokobori et al. (2013).

sterilization by the host and dissemination in the severe forms of tuberculosis, represent dead ends.

Since the advent of molecular epidemiology, *M. tuberculosis* can no longer be regarded as a hyperconserved species. Genetic diversity translates into phenotypic diversity that impacts the outcome of this disease (Henao-Tamayo et al., 2015; López et al., 2003; Manca et al., 1999). Notably, some genotypes manage to spread overcoming strong selective pressures exerted by the host immune system, the environment, and antibiotics (Borrell and Gagneux, 2009). This proved to be true for the M strain, which caused the largest multidrug-resistant (MDR) tuberculosis outbreak in Latin America in the late 1990s (Ritacco et al., 2012a; Ritacco et al., 2012b), despite having accumulated multiple mutations that had rendered it pre-extensively drug resistant (pre-XDR) in the late 1970s. According to its spoligotype, the M strain belongs to the Haarlem family (Table 1), one of the major *M. tuberculosis* clades of the predominant EuroAmerican lineage circulating in Argentina (Ritacco et al., 2012b). Whole genome sequencing analysis showed that some degree of diversification within the M cluster occurred, and marginal branches probably paid a higher fitness cost (Eldholm et al., 2015). This was the case for the non-prosperous 410 strain, a variant of the M strain, contemporary to the outbreak, which caused a single case in two decades despite having numerous opportunities to be transmitted (Yokobori et al., 2013). We have previously shown that the M strain can manipulate macrophage cell death (Yokobori et al., 2012), innate (Yokobori et al., 2013; Yokobori et al., 2012) and adaptive immune responses (Basile et al., 2011; Geffner et al., 2009; Sabio et al., 2017), as well as T cell-mediated cytotoxicity (Geffner et al., 2014), suggesting that it efficiently evades host immunity. However, we still have no direct evidence, other than epidemiology, that can account for the enhanced fitness of the M strain, as its axenic and intracellular growth was slower compared to the laboratory strain H37Rv and 410 (Yokobori et al., 2013). In previous works, we studied isolate 6548, a single representative of the outbreak peak, but the impact of intra-strain variability on the phenotype has not been addressed.

The objective of this study was to further characterize the performance of the outbreak strain M in different fitness assays. Two main aspects were evaluated: (1) molecular and phenotypic characterization of selected isolates of the M outbreak and related strains and (2) comparative fitness of representative M strain isolates vs. the non-prosperous M strain variant 410. A comprehensive analysis of available phenotypic and genotypic data was performed in order to shed light into the bacterial factors that contribute to the epidemiological success of the M strain.

2. Materials and methods

2.1. Clinical isolates

Isolates were selected from the collection belonging to the Mycobacteriology Laboratory, INEI-ANLIS "C. G. Malbrán". All isolates had been recovered from patients with smear positive pulmonary tuberculosis in Buenos Aires city and its metropolitan area, Argentina between 1995 and 2008, and were identified by IS*6110*-based restriction fragment length polymorphism (RFLP) (van Embden et al., 1993) and spoligotyping (Kamerbeek et al., 1997) as belonging to the M prototype strain or closely related genotypes. Drug susceptibility profiles, determined following World Health Organization guidelines (World Health Organization, 2008, 2009), were also taken into account for the selection. Accordingly, isolates were further classified as pan-susceptible to drugs (Pan-S); mono drug-resistant (Mono-R); MDR, defined as resistant to at least rifampicin (RIF) and isoniazid (INH); pre-XDR, defined as MDR plus resistance to either a fluoroquinolone or a second-line injectable drug; and XDR, defined as MDR plus resistance to both a fluoroquinolone and a second-line injectable drug. Isolates 6548, 15526 and 12838 were representative of the prototype M strain obtained at different time points of the outbreak. Other four less closely related isolates were included: (1) the non-prosperous MDR strain 410 which is a M strain variant according to its IS*6110*-RFLP (Yokobori et al., 2013), (2) the isolate 12000 which matches the IS*6110*-RFLP pattern of the prototype M strain but is INH Mono-R, and (3) two isolates, 6145 and 14674, obtained from different patients and representing a Pan-S strain different from the M strain according to RFLP pattern but belonging to the same clade H2 SIT2 according to spoligotyping. These are the closest representatives of a Pan-S "M-like" strain available in our collection (Table 1); no Pan-S isolate matching 100% the M genotype has ever been found. The isolate 6548 and strain 410 were used in all experiments. Other isolates were included in selected experiments. The laboratory strain H37Rv was used as control in all in vitro experiments (kindly provided by I. Kantor, former TB laboratory head, INPPAZ PAHO/WHO). Frozen cultures were recovered in Middlebrook 7H9 broth (BD, USA) with albumin-dextrose

enrichment for 15–21 days. Reculture was kept to a minimum to avoid loss of virulence.

2.2. Molecular characterization

To further characterize the selected isolates, VNTR-MIRU-15 analysis was performed according to international standards (Supply et al., 2006). Canonical mutations conferring drug resistance to RIF and INH were determined by Sanger sequencing of the corresponding regions of *rpoB* gene (primers rpo95/rpo397) (Somoskovi et al., 2003; Telenti et al., 1993), *katG* gene (primers TB86/TB87), and *inhA* promoter (primers TB92/TB93) (Jagielski et al., 2014; Kiepiela et al., 2000). The presence of fluoroquinolone resistance-conferring mutations was investigated by sequencing of the quinolone resistance determining regions in *gyrA* (primers Pri9/Pri8) and *gyrB* (primers GyrbA/GyrbE) (Dauendorffer et al., 2003). Mutated codons were named following H37Rv notation.

2.3. Growth in BACTEC-MGIT system

All the selected isolates and the reference strain H37Rv were evaluated. For axenic growth, a 1:10 dilution of a 0.5 mg/ml bacterial suspension was used to inoculate Mycobacterial Growth Indicator Tubes (MGIT; BD) supplemented with oleic acid-albumin-dextrose-catalase enrichment (BD). Each isolate was cultured in triplicate for 21 days. Multiplication kinetics was monitored in BACTEC 960™ system and BD Epicenter™ software equipped with the *TB eXiST* module (BD). Growth curves were depicted as growth units (arbitrary units used to measure growth in the BACTEC-MGIT system) vs. time (Fig. 1). Lag phase length, doubling time, time to stationary phase and growth units in the stationary phase were calculated as described elsewhere (von Groll et al., 2010), using growth units as surrogate of viable bacilli counts. To assess growth in the presence of antibiotics, selected strains were cultured in MGIT containing INH and RIF at the concentrations recommended for susceptibility testing in this system (0.1 and 1.0 μg/ml respectively). In addition, for the isolates selected for this latter assay, minimal inhibitory concentrations (MIC) for INH and RIF were determined by the resazurin microtiter assay, as previously described (Martin et al., 2005). Briefly, serial two-fold dilutions of each drug were prepared in 96-well microtiter plates using 7H9-oleic acid-albumin-dextrose-catalase enrichment. The concentration ranges tested were 0.006-12.0 μg/ml for INH, and 80–0.625 μg/ml, for RIF. Two growth controls containing no antibiotic and a sterile control were also prepared on each plate. After 7 days, 30 μl of resazurin solution was added and the plate was reincubated overnight. The MIC was defined as the lowest drug concentration that prevented the change in color due to bacterial activity. Isolates with MIC values ≥ 0.25 μg/ml for INH and ≥ 0.5 μg/ml for RIF were considered resistant.

2.4. Human macrophage differentiation and infection

Human monocyte-derived macrophages (MDM) were obtained from buffy coats of 7 healthy volunteers who gave written informed consent (Regional Center of Hemotherapy, Garrahan Hospital, Buenos Aires, Argentina). Exclusion criteria included positive serology for HIV, hepatitis B, syphilis and Chagas disease. The Ethics Committee of the Academia Nacional de Medicina approved all experimental procedures in accordance with the Helsinki Declaration. Briefly, peripheral blood monocytes were purified in Ficoll-Hypaque and Percoll (GE Healthcare, Sweden) gradients as described elsewhere (Hardin and Downs, 1981), plated in 96-well plates and allowed to differentiate to macrophages in 5% CO_2 at 37 °C for 7 days in RPMI 1640 medium (EMEVE, Argentina) supplemented with 10% fetal calf serum (Natocor, Argentina) and 2 mM L-glutamine (EMEVE), hereafter mentioned as complete medium. Purity and viability were routinely checked. For MDM infection, a fresh 7H9-albumin-dextrose culture of each strain was harvested in late

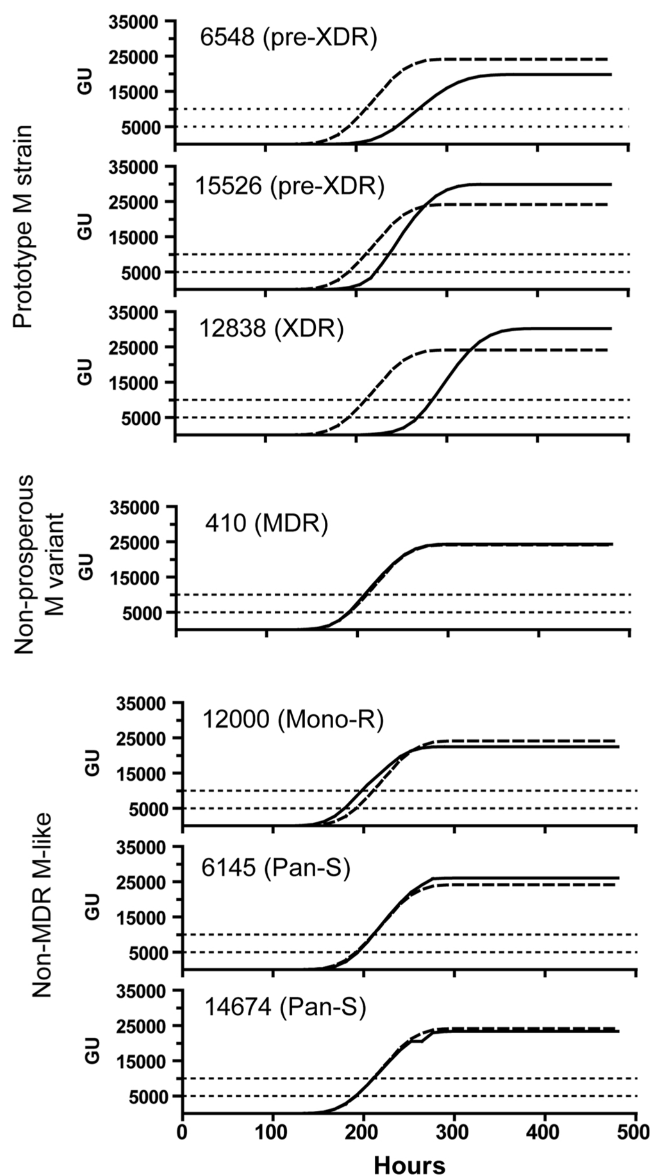

Fig. 1. Growth curves of the selected isolates in the BACTEC-MGIT system. Data are expressed as growth units (GU) vs. time (in hours). Each isolate was assessed in triplicate, and the average curve for each is shown. Doubling time in exponential phase was arbitrarily calculated between 5000 and 10000 GU, represented by discontinuous lines in Y axis (Table 3). Each panel represents the growth curves corresponding to the strain indicated in the inset (solid line) and H37Rv strain (dashed line). Abbreviations: pre-XDR: pre-extensively drug-resistant; XDR: extensively-drug resistant; MDR: multidrug-resistant; Mono-R: mono drug-resistant; Pan-S: pan-susceptible.

exponential phase. After a water bath sonication cycle, clumps were removed by low speed centrifugation. Concentration was adjusted according to 600 nm optical density and colony forming units (CFU)/ml were checked by serial dilution plating onto 7H11-albumin-dextrose. MDM were infected in triplicate with a multiplicity of infection (MOI) of 1 bacterium per cell for 1 h, to avoid macrophage cytotoxicity. Real MOI ranged from 0.5 to 2. Intracellular replication was measured by counting CFU at days 0, 2 and 4 after infection. MDM were lysed with a 0.05% Triton X-100 solution in phosphate buffered saline and CFU were determined by serial dilution and plating. Plates were incubated at 37 °C in 5% CO_2 for 2–5 weeks until colonies became visible and countable.

2.5. Competition assays

Competition assays were performed in both axenic culture (day 0 and day 21) and intracellular conditions (days 0, 2 and 4 post infection). Equal parts of competitor strains were mixed and used to inoculate MGIT tubes or to infect MDM as described. CFU were counted by serial dilution and plating onto 7H11-albumin-dextrose with or without antibiotics to discriminate the strains. Kanamycin (KAN; 6 μg/ml) was used for 6548 or 15526 vs. 410 and RIF (1 μg/ml) was used for 6548 or 15526 or 410 vs. H37Rv (Table I). Inocula were similarly checked (data not shown).

2.6. Mouse infection

All the experiments involving mouse infection were conducted at the INCMNSZ, Mexico, in accordance with the National Regulations on Animal Care and Experimentation. A previously described murine model of progressive pulmonary tuberculosis was used (López et al., 2003). Briefly, 6–8 week BALB/c mice were infected intratracheally with 1×10^6 CFU. Two groups of 50 mice each were infected with isolate 6548, representative of the M outbreak peak, and the non-prosperous strain 410. Eight mice (all survivors, if less than eight) were killed by exsanguination at 3, 14, 21, 28, 60 and 120 days after infection. Lungs were prepared for histopathological and microbiological studies as previously described (Dormans et al., 2004). For CFU counts, lungs were homogenized and CFU/ml were determined by serial dilution and plating. Histopathology and morphometry were analyzed in haematoxilin and eosin-stained paraffin sections of the lungs. The area in μm^2 corresponding to the interstitial and perivascular inflammation, as well as the percentage of lung surface affected by granulomas and pneumonia, were determined using an automated image analyser (Q Win Leica, Milton Keynes, UK) (Hernandez-Pando et al., 2000). Delayed-type hypersensitivity (DTH) was assessed as described elsewhere (Dormans et al., 2004).

2.7. Statistics

A one-way ANOVA and Student's *t*-test were used to compare axenic growth, morphometry and CFU counts recovered from mice. MDM responses were evaluated by the nonparametric Friedman test, followed by Wilcoxon paired test to compare two groups. Statistical analysis of survival curves was performed using Kaplan-Meier plots and Long Rank tests. A value of $P < .05$ was assumed as significant.

3. Results

3.1. Isolate characterization

First, we studied the 15-loci MIRU-VNTR as an additional genetic marker among the selected isolates (Table 1). MIRU-VNTR loci were highly conserved within the prototype M cluster with very few changes. Even the non-prosperous strain 410 and M-like isolates showed minimal differences with the MIRU-VNTR pattern of the M strain, confirming their relatedness with the outbreak isolates. No isolates sharing the MIRU-VNTR pattern of 410 strain was found in our collection, endorsing the singularity of this genotype.

Next, we searched for polymorphisms in selected genes related to drug resistance (Table 2). All INH-resistant isolates had a common mutation in *katG* gene, and no mutation in *inhA* as previously described (Eldholm et al., 2015). All isolates in the M outbreak shared the S450L mutation in *rpoB*, and Q10P mutation in *pncA*; the XDR isolate 12838 had additional mutations in *gyrB* and *gyrA* loci, in accordance with its fluoroquinolone resistance. In turn, the non-prosperous strain 410 owed its RIF resistance to an H445L *rpoB* mutation and its PZA resistance to a Y103D mutation in *pncA* (Bigi et al., 2017). The other isolates were wild type for the tested genes.

3.2. Axenic growth and response to antibiotics

The ability of the selected isolates to grow in culture, the simplest measure of bacterial fitness (Wiser and Lenski, 2015), was assessed in the BACTEC-MGIT system (Fig. 1). The reference strain H37Rv was used as control. Isolate 6548 had a significantly longer doubling time, which led to lower growth units at the end of the assay (Fig. 1, Table 3). This was not observed for later M isolates; both isolate 15526 and even the XDR isolate 12838, had a shorter doubling time than 6548. All three isolates of the M outbreak had long lag phases, and this was exacerbated in the XDR strain. Neither the lag phase nor the replication rate of the unique strain 410 were compromised ($P > .05$). The growth curves of the Pan-S and the Mono-R isolates were identical to that of H37Rv (Fig. 1 and Table 3).

Next, the growth kinetics of the MDR strains in the presence of RIF and INH was evaluated (Table 3). The presence of RIF plus INH did not retard the growth of the M isolates; what is more, it rather boosted the replication of isolate 6548. Conversely, the growth of 410 was severely impeded in the presence of INH plus RIF. In a different experiment, we determined that RIF was the drug responsible for such interference (data not shown). In accordance, M strain isolates had a higher MIC for RIF than 410 strain, while all strains had equal resistance levels to INH (Table 2).

3.3. Competition assays

To determine relative fitness, first we performed competition assays in axenic culture. Taking into account individual growth patterns, isolates 6548 (outbreak peak, slow axenic growth), 15526 (late outbreak, growth rate not compromised), and 410 (non-prosperous) were selected, along with the laboratory strain H37Rv. The following combinations were evaluated: 6548 vs. 410, 15526 vs. 410, 6548 vs. H37Rv, 15526 vs. H37Rv, 410 vs. H37Rv. Our results show that both isolates belonging to the M outbreak outgrew the 410 strain (Fig. 2A, upper panels). However, H37Rv was the fittest compared to all clinical isolates.

Next, we evaluated their relative fitness in the intracellular niche. MDM infected with individual strains served as controls. As shown in Fig. 2B and Supplementary Fig. 1, individually, M isolates and 410 replicated more efficiently than H37Rv intracellularly (*$P < .05$; Wilcoxon paired test), with no significant differences between them. In intracellular competition, no clear predominance of any strain over the competitor was observed.

3.4. Murine model of progressive pulmonary tuberculosis

The M outbreak isolate 6548 and the non-prosperous variant 410 were selected for in vivo infection in a murine model of progressive pulmonary tuberculosis, according to the following criteria: 1) previous results regarding host immune response against the M strain were obtained with isolates 6548 and 410 (Basile et al., 2011; Geffner et al., 2014; Geffner et al., 2009; Yokobori et al., 2013; Yokobori et al., 2012); 2) they are contemporaneous strains (Table 1), representative of the outbreak peak; 3) despite its slower growth in culture, 6548 showed higher fitness than 410 in competition and in the presence of antibiotics.

Fig. 3A shows that no significant differences in mortality were observed among the selected strains (Fig. 3A). In spite of this, the 410 strain replicated more vigorously than 6548 in the lungs (Fig. 3B).

Regarding histopathology, 410 induced stronger interstitial and perivascular inflammation, which was the earliest manifestation, and more organized granulomas, which were paralleled by a stronger DTH response (Fig. 4). Pneumonic areas appeared later, increased steadily until the end of the experiment, and were similar in both strains (Fig. 4C). Remarkably, by the end of the assay, 6548 induced more interstitial inflammation than 410 despite the lower bacillary burden

Table 2
Phenotypic drug resistance profile of the selected strains and the corresponding resistance-related mutations

Isolate n°/ year.	Drug susceptibility profile								Resistance-conferring mutations								
	Profile	INH (MIC in µg/ml)	RIF (MIC in µg/ ml)	STR	EMB	PZA	KAN	OFX	*katG*	*inhA*	*rpoB*	*gidB*	*embB*	*pncA*	*rrs*	*gyrA*	*gyrB*
Reference strain																	
H37Rv	Pan-S	S (0.025)	S (< 0.625)	S	S	S	S	S	WT	WT	WT	WT	WT	WT	WT	WT	WT
Prototype pre-XDR/XDR M strain isolates																	
6548/98	Pre-XDR	R (3.15)	R (> 80)	R	R	R	R	S	S315T	WT	S450L	V110 fs[a]	G406A[a]	Q10P[a]	1401 A > G[a]	WT	WT
12838/05	XDR	R (3.15)	R (> 80)	R	R	R	R	R	S315T	WT	S450L	V110 fs[a]	G406A[a]	Q10P[a]	1401 A > G[a]	D94G	A504V
15526/07	Pre-XDR	R (3.15)	R (> 80)	R	R	R	R	S	S315T	WT	S450L	V110 fs[a]	G406A[a]	Q10P[a]	1401 A > G[a]	WT	WT
MDR unique M-related strain																	
410/95	MDR	R (3.15)	R (20)	R	S	R	S	S	S315T	WT	H445L	V110 fs[b]	WT[b]	Y103D[b]	WT[b]	WT[b]	WT[b]
Non MDR M-related strains																	
12000/04	Mono-R	R	S	S	S	S	S	S	S315T	WT	WT	ND	ND	ND	ND	ND	ND
6145/98	Pan-S	S	S	S	S	S	ND	ND	WT	WT	WT	ND	ND	ND	ND	ND	ND
14674/06	Pan-S	S	S	S	S	S	ND	ND	WT	WT	WT	ND	ND	ND	ND	ND	ND

Abbreviations: MIC: minimal inhibitory concentration; INH: isoniazid; RIF: rifampicin; STR: streptomycin; EMB: ethambutol; PZA: pyrazinamide; KAN: kanamycin; OFX: ofloxacin; MDR: multidrug-resistant; XDR: extensively drug-resistant; Mono-R: mono drug-resitant; Pan-S: pansusceptible; S: susceptible; R: resistant; ND: not determined; WT: wild type; fs: frame shift. INH and RIF MIC values of the isolates/strains cultured in the presence of these drugs (Table 3)and the reference strain H37Rv are shown. Resistance conferring mutations in *gidB*, *embB*, *pncA* and *rrs* were added for comparison and were published in [a]Eldholm et al. (2015) and in [b]Bigi et al. (2017).

Table 3
Axenic growth kinetics.

Isolate/strain	Duration of lag phase (hours)		Doubling time in exponential phase (hours)		Time to stationary phase (hours)		Growth units at stationary phase	
	–	+INH + RIF	–	+INH + RIF	–	+INH + RIF	–	+INH + RIF
Reference strain								
H37Rv	122.8 ± 0.7	–	19.5 ± 0.5	–	320	–	24159 ± 426	–
Pre-XDR M isolates								
6548	166.5 ± 1.0†	172.0 ± 0.4	24.5 ± 1.0**	20.4 ± 0.4c	382	374	19821 ± 801a	22846 ± 474d
15526	157.9 ± 0.3†	160.7 ± 1.0	14.5 ± 0.3*	14.0 ± 0.4	341	341	29867 ± 674b	30487 ± 674
XDR M isolate								
12838	194.1 ± 0.4‡	192.1 ± 2.7	16.5 ± 0.5	16.0 ± 0.3	395	396	30205 ± 714	31154 ± 549
Non-prosperous MDR M strain variant								
410	120.9 ± 0.3	382.9 ± 6.6	17.7 ± 0.8	31.5 ± 1.5d	304	746	24324 ± 884	16597 ± 737d
M-like Mono-R isolate								
12000	112.8 ± 0.5	–	19.0 ± 0.6	–	289	–	22459 ± 595	–
M-like Pan-S isolates								
6145	124.7 ± 0.1	–	17.4 ± 0.6	–	311	–	26037 ± 558	–
14674	123.0 ± 0.4	–	19.8 ± 0.6	–	307	–	23353 ± 592	–

Abbreviations: INH: isoniazid; RIF: rifampicin; MDR: multidrug-resistant; XDR: extensively drug resistant; Mono-R: mono drug-resistant; Pan-S: pansusceptible. Duration of lag phase and time to stationary phase were defined as the time to culture positivity and time to plateau, both in hours of culture, in the BACTEC-MGIT system. Doubling time was calculated as previously described (von Groll et al., 2010) using growth units form BACTEC-MGIT system as surrogate of viable bacilli counts. Growth in the presence of INH plus RIF was performed with a final concentration of 0.1 and 1.0 µg/ml respectively. Data are represented as mean ± SEM of triplicates. Statistical significances: [†]P < .001, 6548/15526 vs. others; [‡]P < .001 12878 vs. others; **P < .01 6548 vs. other strains/isolates; *P < .05 12838 vs. 15526;[a]P < .05 6548 vs. others; [b]P < .01 15526 vs. H37Rv; [c]P < .0001 vs. w/o INH/RIF; [d]P < .01 vs. w/o INH/RIF.

(Fig. 3B).

4. Discussion

Although the obligate pathogenic lifestyle of *M. tuberculosis* imposes a number of constrains to its ability to evolve, both host immunity and the strong selective pressure exerted by antibiotics force this species to adapt to the changing environment. The availability in a particular place and time of numerous HIV positive hosts, a very fertile niche for its spread, probably catalyzed the clonal expansion of this highly successful MDR strain (Eldholm et al., 2016). Still, the results described herein indicate that certain intrinsic characteristics of the M strain might also account for its formidable epidemiological success.

The hypothetical phylogenetic relationships among the isolates included in this study are summarized in the Supplementary Fig. 2. The 15-loci MIRU-VNTR pattern of the prototype M strain, herein represented by isolates 6548 and 12838, has been validated in our laboratory with a large number of isolates (data not shown). MIRU-VNTR patterns of the other isolates in this study were fairly conserved (Table 1), what speaks eloquently of the genetic stability of these markers in the M progeny throughout more than four decades of parallel evolution (Eldholm et al., 2015).

Differences in the ability to adapt and grow in optimal culture conditions usually uncover variations in crucial components of bacterial biology. All the included isolates were able to grow in axenic culture, but interesting differences in their kinetics were observed. The growth curves of the Pan-S and the Mono-R isolates overlapped tightly with the curve of H37Rv (Fig. 1) suggesting that they are reasonably fit.

A.

6548 vs. 410 — 15526 vs. 410 — 6548 vs. H37Rv — 15526 vs. H37Rv — 410 vs. H37Rv

CFU/ml; D0, D21

B.

6548 vs. 410 — 15526 vs. 410 — 6548 vs. H37Rv — 15526 vs. H37Rv — 410 vs. H37Rv

Median CFU/ml; D0, D2, D4

Fig. 2. Competition assays. A. Competition in axenic culture. MGIT tubes were inoculated with equal parts of the indicated strain/isolate alone (solid lines and symbols) or in competition (c; dashed lines and open symbols). CFU/ml were determined at day 0 and day 21 post-inoculation. Results are expressed as mean and standard error of mean of triplicates. One representative experiment of three performed is shown. **B.** Intracellular competition. Human monocyte derived macrophages were infected with the indicated strain/isolate alone (solid lines and symbols) or in competition (c; dashed lines and open symbols) at a multiplicity of infection of 1. CFU/ml of the lisates were determined at day 0, 2 and 4 post-infection. In order to facilitate visual comparison with Fig. 2A, results are expressed as the median of 7 experiments. Lower panels: 6548/15526/410 vs. H37Rv in single infections: *P < .05 (Wilcoxon paired test). A detailed description of these results and statistical analysis are included in Supplementary Fig. 1.

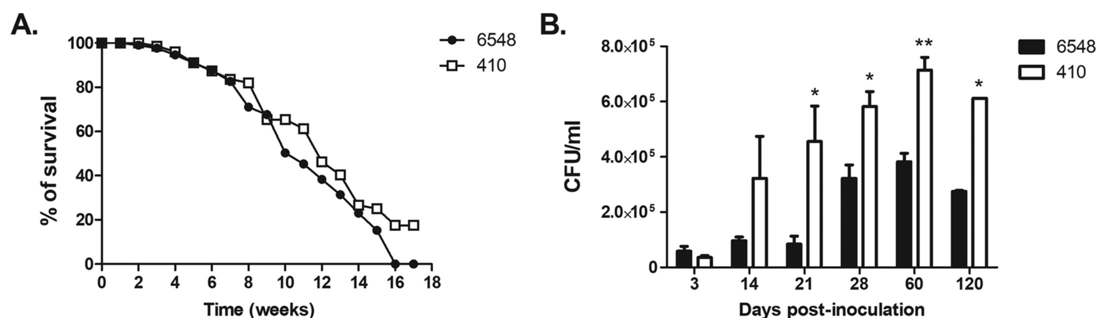

Fig. 3. In vivo infections. A. Survival of BALB/c mice infected by intratracheal inoculation of 1×10^6 CFU of isolate 6548 of the prototype M strain (solid symbols) and the non-prosperous M strain variant 410 (open symbols). No significant differences in survival were observed. **B.** CFU/ml in lung homogenates during the course of infection with M strain isolate 6548 (solid bars) or 410 strain (open bars). Statistical significances: for 6548 vs. 410, *P < .05; **P < .01; Student's *t*-test. Data represent the average of three independent experiments.

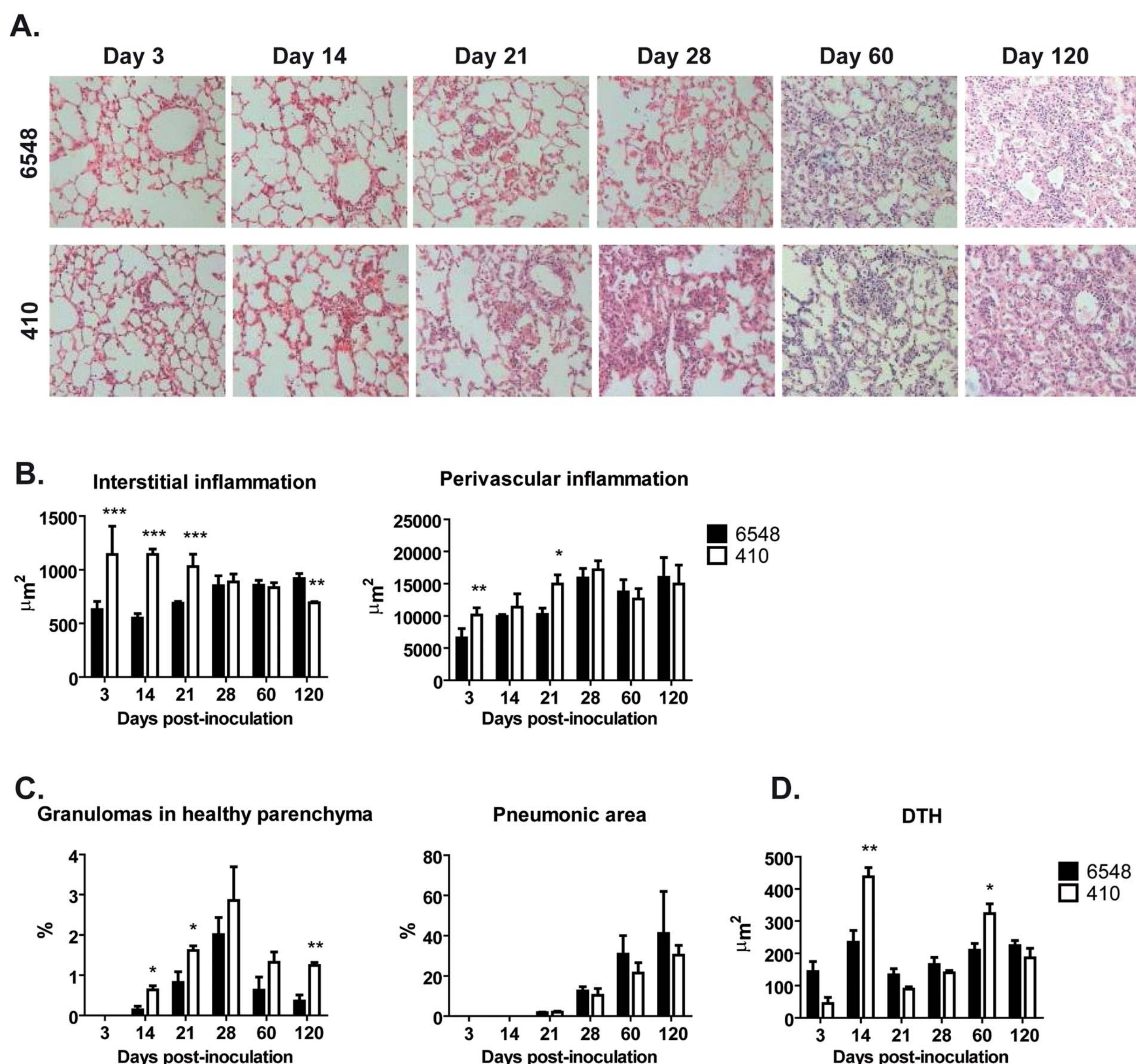

Fig. 4. Lung histopathology and immune response. A. Histopathology analysis of haematoxylin-eosin stained transverse sections of lungs collected at the indicated time points after infection (40× magnification) from mice infected with isolate 6548 (upper panels) or 410 strain (lower panels) **B.** Morphometric analysis of the inflammatory infiltrate observed during the course of infection in the lung parenchyma and perivascular area. Three random fields for each lung lobe were analyzed. Data are represented as affected surface in μm^2 at the indicated times. **C.** Percentage of lung surface affected by granulomas and pneumonia. **D.** Delayed type hypersensitivity (DTH) in response to intracutaneous injection of *M. tuberculosis* culture filtrate antigens. Data represent foot-pad swelling in μm^2 at the indicated time points. Statistical significances: $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$; Student's *t*-test.

However, our results also show that the relationship between epidemiological success and axenic growth ability might not be straightforward. The accumulation of resistance-conferring mutations altered the growth curves of M outbreak isolates, and this contrasted with the apparently unaltered growth ability of the non-prosperous 410 strain. On the other hand, isolate 6548, representative of the peak of the M outbreak, had compromised its ability to adapt and to replicate in vitro. Interestingly, although the longer lag phase of all three tested isolates of the M cluster was conserved, the longer replication rate was a hallmark of isolate 6548.

Noticeably, the outward in vitro growth advantage of 410 strain over the outbreak isolates was reversed under antibiotic pressure (Table 3). This result is likely to be related to the H445L substitution in *rpoB* harbored by the non-prosperous 410 strain. This mutation has been associated with discordant or borderline RIF resistance (Angra et al., 2012; Hauck et al., 2009; Ocheretina et al., 2015; Van Deun et al., 2009; Williamson et al., 2012) and eventual misdiagnosis (Van Deun et al., 2009). In addition, this substitution is less frequently found among RIF resistant clinical isolates (Boritsch and Brosch, 2016; Ocheretina et al., 2014), suggesting a higher fitness cost (Gagneux et al., 2006). 410 strain had a robust resistance to RIF, with a MIC value 40 fold higher than the cutoff value, but its growth disadvantage in the presence of INH and RIF correlated with a > 4 times lower RIF MIC value compared to M strain isolates (Tables 2 and 3). Thus, the presence of this drug, even at low concentrations, constituted a stressful condition to the non-prosperous 410 strain, in accordance with the epidemiologically inferred fitness cost imposed by H445L *rpoB* mutation.

The underlying biology of the uneven occurrence of different *rpoB* mutations are largely unknown (Koch et al., 2014), but isolates in the prototype M strain harbor the most frequently found mutation H450L, which allegedly bears minimal or null fitness cost. In contrast with the 410 strain, isolates in the M outbreak showed a higher resistance to RIF, and isolate 6548 even had some advantage in the presence of the drugs (Table 3). Interestingly, a RIF-enhanced isolate carrying S450L and F503S mutations in *rpoB* was described, which also showed improved growth in the presence of RIF (Zhong et al., 2010). Isolate 6548 carries no secondary mutations in *rpoB* but an unusual mutation in *rpoC* (INS739P), which is absent in other M isolates (Bigi et al., 2017;

Eldholm et al., 2015), and could be responsible for a similar RIF-dependent enhanced growth.

Axenic competition assays further unveiled the advantage of the M strain isolates over the non-prosperous strain 410, and this was most noticeable for isolate 6548 due to its poor individual growth. In this line, some drug resistant isolates with longer lag phase were described previously (Toungoussova et al., 2004) but this trait did not necessarily correlate with poor fitness (von Groll et al., 2010). Conversely, the long time required by M isolates for adaptation to the culture medium might explain in part the predominance of H37Rv in competition. Actually, it is not surprising that the laboratory strain, which was successively passed since its isolation more than a century ago (Ioerger et al., 2010), was the best competitor in the artificial culture medium, as previously reported (Bhatter et al., 2012; Naidoo and Pillay, 2014). Intracellular competition assays have been reported by other authors to be useful to evaluate the relative fitness of clinical isolates (Barczak et al., 2005; Navarro et al., 2013), but our results were not conclusive. Further evaluation of different conditions, including shorter assays with higher MOI to force the competition between strains inside the macrophages, might be informative. Nevertheless, from our results we can conclude that the tested strains/isolates are at least similarly fit in these conditions. In line with this, the isolate 6548 showed an individual intracellular growth rate comparable to the other isolates, despite its slow replication in axenic conditions. This is particularly interesting taking into account that bacilli recovered from the intracellular milieu had the same slow growth upon reculture on agar plates (Supplementary Fig. 3). This intriguing trait of 6548 suggests that it is highly adapted to intracellular survival, but behaves clumsy outside the cell.

Our results with the Balb/c mouse model of progressive pulmonary infection showed that, in line with results obtained with other Haarlem family strains, both 6548 and 410 were less virulent than H37Rv, which induced 100% mortality at week 12 in previous assays performed in equal conditions (López et al., 2003). Bacterial burden partially recapitulated the behavior of these strains in individual culture, with higher CFU counts for 410 strain. Overall, 410 strain induced stronger inflammatory and DHT responses, mainly at the early stage, along with better formed granulomas, indicative of a protective response (Gideon and Flynn, 2011). This was in contrast with a more discrete granuloma formation with diffuse cellular infiltrates induced by 6548, along with pneumonia and higher interstitial inflammation at the later stage. Both strain variants induce a similarly low mortality, but distinct histopathology and inflammatory responses. Considering that lung histopathology, virulence and bacterial burden usually correlate in this in vivo model (Dormans et al., 2004), the observed dissociation with bacillary loads herein observed is remarkable. We have previously observed that the 410 strain resembled CDC1551 strain in its cytokine induction pattern (Yokobori et al., 2013). The CDC1551 was known for the unusually high skin test conversion rate among contacts, and proved to be able to induce a prompt and well formed granulomas in lungs of infected mice (Manca et al., 1999). Unfortunately, PPD skin test status among contacts of 410 strain are not available, but our histopathological and DTH test results of experimental infection support the hypothesis that this strain could have been readily transmissible but those infected individuals would have not progressed to disease. Despite the phylogenetic distance between CDC1551 (which belongs to the X3 spoligotype, SIT 549) and 410 strain, the convergence in the murine infection outcome is remarkable.

The divergent phenotypes of isolate 6548 and its non-prosperous close relative 410 described herein would stem from key genomic differences. We have previously described that the additional IS*6110* copy present in 410 strain is inserted in the Rv3638/Rv3639c intergenic region (Reyes et al., 2012) but the biological relevance of this transposition event remains unknown. Pairwise comparison of the genomes of isolates 6548 and 410 showed differences in regions related to virulence, acquisition of metal residues and lipid biosynthesis (Bigi et al., 2017). Moreover, drug resistance conferring mutations might have epistatic effects (Borrell and Gagneux, 2011; Koch et al., 2014) that can translate into deep metabolic reprogramming even in the absence of the drug (Lahiri et al., 2016). Integration of the current and previous results with ongoing transcriptomic and lipidomic studies is of crucial importance to understand the mechanisms underlining their phenotypic differences.

5. Conclusions

Our approach evaluating various aspects of *M. tuberculosis* fitness confirms its multifaceted nature, as the different outcomes of individual assays might lead to divergent conclusions. Altogether, we can conclude that the epidemiologically abortive strain 410 was poorly resistant to drug-driven stress, a weak competitor and a stronger inducer of protective response in vivo. Conversely, the isolate representative of the M outbreak peak (6548) had a clear growth disadvantage but performed very well in competition and induced lung damage at advanced stages of the experimental infection despite reaching relatively low CFU counts. Integration of these observations supports the idea that the M strain managed to find a unique path to success which was probably already grounded early in its evolution.

Potential conflicts of interest

All authors declare no conflicts of interest.

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Author contributions statement

N.Y., B.L., J.C.P., R.H.P., M.C.S. and V.R. conceived and designed the experiments; N.Y., B.L., A.V.G., A.M. and B.M.C. performed the experiments. J.M. and R.P. performed the genotyping assays, evaluation of MICs and analysis. N.Y., B.L., R.H.P. analyzed the results. N.Y., B.L., J.C.P., R.H.P., M.C.S. and V.R. provided resources and materials. N.Y., B.L. and V.R. wrote the manuscript. All authors reviewed and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijmm.2018.01.006.

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