

Genome-wide screen identifies host loci that modulate *Mycobacterium tuberculosis* fitness in immunodivergent mice

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Abstract

Genetic differences among mammalian hosts and among strains of *Mycobacterium tuberculosis* (*Mtb*) are well-established determinants of tuberculosis (TB) patient outcomes. The advent of recombinant inbred mouse panels and next-generation transposon mutagenesis and sequencing approaches has enabled dissection of complex host–pathogen interactions. To identify host and pathogen genetic determinants of *Mtb* pathogenesis, we infected members of the highly diverse BXD family of strains with a comprehensive library of *Mtb* transposon mutants (TnSeq). Members of the BXD family segregate for *Mtb*-resistant C57BL/6J (B6 or B) and *Mtb*-susceptible DBA/2J (D2 or D) haplotypes. The survival of each bacterial mutant was quantified within each BXD host, and we identified those bacterial genes that were differentially required for *Mtb* fitness across BXD genotypes. Mutants that varied in survival among the host family of strains were leveraged as reporters of “endophenotypes,” each bacterial fitness profile directly probing specific components of the infection microenvironment. We conducted quantitative trait loci (QTL) mapping of these bacterial fitness endophenotypes and identified 140 host–pathogen QTL (*hpQTL*). We located a QTL hotspot on chromosome 6 (75.97–88.58 Mb) associated with the genetic requirement of multiple *Mtb* genes: *Rv0127* (*mak*), *Rv0359* (*rip2*), *Rv0955* (*perM*), and *Rv3849* (*espR*). Together, this screen reinforces the utility of bacterial mutant libraries as precise reporters of the host immunological microenvironment during infection and highlights specific host–pathogen genetic interactions for further investigation. To enable downstream follow-up for both bacterial and mammalian genetic research communities, all bacterial fitness profiles have been deposited into GeneNetwork.org and added into the comprehensive collection of TnSeq libraries in MtbTnDB.

Keywords: host–pathogen interactions, tuberculosis, mycobacteria, systems genetics, genomics, genetic diversity, natural variation, bacterial genetics, TnSeq, mouse models, BXD, QTL mapping, complex traits

Introduction

Every pathogenic infection is part of an evolutionary battle between host and invader. In the case of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), evidence tracing back at least 9,000 years tells the tale of a host–pathogen arms race (Hershkovitz et al. 2008), making *Mtb* one of the most enduring adversaries of the human species. Given the prevailing nature of this challenge, it is no surprise that weaknesses in vital host defenses provide greater opportunity for *Mtb* infection, allow *Mtb* to manipulate the nature and magnitude of the host immune response, and worsen patient prognoses (Cooper et al. 1993; Flynn et al. 1993; Cooper et al. 1997; Caruso et al. 1999; Kramnik et al. 2000). In addition, the advent of drug-resistant and multidrug-resistant *Mtb* strains highlights the imminent danger posed by our microscopic rivals and underscores the importance

of bacterial variation as a critical factor in the pathogenesis and continued spread of *Mtb* (Caws et al. 2008; Hernández-Pando et al. 2012; Gopal et al. 2014). Meanwhile, genetic diversity of both hosts and *Mtb* strains that interact during infection can give rise to a spectrum of disease outcomes (Smith and Sasseti 2018), enhancing the complexity of TB identification and treatment. To combat a pathogen responsible for nearly 10.6 million infections and 1.6 million deaths annually (WHO 2022), it is therefore vital to dissect the host–pathogen interface.

Mammalian models of TB have been used successfully to identify and mechanistically interrogate TB susceptibility loci identified from human cohorts. For mouse strains that are phenotypically divergent, quantitative trait locus (QTL) mapping studies within genetically tractable and reproducible hosts have enabled the identification of genomic loci that contribute to the magnitude

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of clinically relevant phenotypes (Lavebratt *et al.* 1999; Kramnik *et al.* 2000; Mitsos *et al.* 2000; Mitsos *et al.* 2003; Yan *et al.* 2006). The classic inbred strains C57BL/6J (B6) and DBA/2J (D2) lie on opposite ends of the TB susceptibility spectrum, with B6 surviving nearly a year postinfection and D2 succumbing to cachexia and morbidity within months (Medina and North 1998). To capitalize on this diverse disease outcome and just over 6 million divergent SNPs (Wang *et al.* 2016; Ashbrook *et al.* 2021; Ashbrook *et al.* 2022; Sasani *et al.* 2022), a recombinant inbred panel generated from these 2 founders, known as the BXD, has laid the foundation for understanding host determinants of susceptibility to a variety of diseases through QTL mapping in genetically diverse mice (Taylor *et al.* 1973; Taylor *et al.* 1999; Peirce *et al.* 2004; Ashbrook *et al.* 2021).

BXD and other recombinant inbred panels have been used to explore the intricate dynamics of TB disease, but often the phenotypes leveraged to understand these dynamics in mice, such as survival time postinfection, body weight, or bacterial burden, are too complex and polygenic to reveal novel biology or enable refined mapping. These “macrophenotypes” materialize from many genetic and environmental factors that can be difficult to mechanistically dissect or precisely map. Within each unique host, bacteria face similarly diverse immunological dynamics and spatiotemporal selective pressures. To contribute to the growing body of work on genetic determinants of TB outcome, quantifying precise bacterial “endophenotypes” underlying disease outcomes is the crucial next step toward elaborating the biological intricacies that govern the host–pathogen interface. Recently employed molecular endophenotypes include expression QTL (Chesler *et al.* 2005), metabolite QTL (Wu *et al.* 2014), protein QTL (Chick *et al.* 2016), and chromatin accessibility QTL (Skelly *et al.* 2020), but for infectious disease, the most salient endophenotypes lie at the interface of the host and pathogen.

A classic approach to assess the requirement of a bacterial gene for infection is to infect a standard inbred mouse strain, such as B6, with a single knockout *Mtb* mutant. Expanding on this design, genome-wide mutagenesis enables the generation of single knockout mutants across the *Mtb* genome, thereby allowing the measurement of *Mtb* gene requirements under a variety of selective pressures. Transposon (Tn) mutant libraries (TnSeq) have been successfully used to report on variable components of the bacterial infection microenvironment under distinct host and antibiotic pressures (Sasseti and Rubin 2003; Nambi *et al.* 2015; Mishra *et al.* 2017; Bellerose *et al.* 2019). We have recently leveraged TnSeq across genetically diverse Collaborative Cross (CC) mice to map both host- and pathogen-linked loci (Smith *et al.* 2022). Deep phenotyping of disease traits in over 50 CC strains enabled mapping of 10 tuberculosis immunophenotype QTL (TipQTL), derived from bacterial burden and cytokine quantifications in each host. Measurement of TnSeq mutant fitness during *in vivo* infection yielded 46 host interacting with pathogen QTL (HipQTL). While 8 founder lines do provide a greater extent of SNP-level diversity in the CC panel than in the BXD panel (Munger *et al.* 2014), the additional founders can reduce statistical strength for QTL mapping and complicate determination of allele effects for mapped QTL. The 2-state model of the BXD panel allows relatively straightforward identification of significant QTL and downstream allele effects, potentially shortening the path from screen to biological discovery.

To further characterize the BXD phenome (Chesler *et al.* 2004) and add to the growing repertoire of selective pressures applied to *Mtb* whole-genome mutant libraries (Jinich *et al.* 2021), we now leverage the phenotypic divergence and reproducibility of

the BXD family and the molecular phenotyping precision of an *Mtb* Tn mutant library. We infected 19 BXD strains and both parents with a saturated library of *Mtb* Tn mutants and conducted dual-genome QTL mapping to identify linkages between bacterial fitness profiles and host genetic factors, revealing 140 host genetic loci significantly associated with specific bacterial mutants. Thus, in passing a bacterial Tn mutant library through selection within the immunologically and phenotypically diverse BXD microenvironments, we have conducted a multidimensional host–pathogen screen to identify precise host and bacterial determinants of TB disease. Overall, this study provides a blueprint for the dissection of host–pathogen interactions, which can play a pivotal role in functional annotation of understudied host and bacterial genes.

Materials and methods

Ethics statement

All mouse studies were conducted in accordance with the guidelines issued in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare. Animal studies conducted at Duke University Medical School were conducted using protocols approved by the Duke Institutional Animal Care and Use Committee (IACUC) (Animal Welfare Assurance #A221-20-11) in a manner designed to minimize pain and suffering in *Mtb*-infected animals. Any animal exhibiting signs of severe disease were immediately euthanized in accordance with IACUC-approved endpoints. All mouse studies conducted at the University of Massachusetts Medical School (UMass) were performed using protocols approved by UMass IACUC (Animal Welfare Assurance #A3306-01).

Mice

Male and female C57BL/6J (#000664) and DBA/2J (#000671) mice were purchased from The Jackson Laboratory. Male mice from 19 BXD strains were imported from the colony of Robert Williams (University of Tennessee Health Science Center, Memphis, TN, USA) in 2013. The 19 BXD strains in this study include BXD9/TyJ, BXD29/Ty, BXD39/TyJ, BXD40/TyJ, BXD48a/RwwJ, BXD51/RwwJ, BXD54/RwwJ, BXD56/RwwJ, BXD60/RwwJ, BXD62/RwwJ, BXD67/RwwJ, BXD69/RwwJ, BXD73/RwwJ, BXD73b/RwwJ, BXD77/RwwJ, BXD79/RwwJ, BXD90/RwwJ, BXD93/RwwJ, and BXD102/RwwJ. All mice were housed in a specific pathogen-free facility within standardized living conditions (12-h light/dark, food and water *ad libitum*). Mice were matched at 8–12 weeks of age at the time of *Mtb* infection. Male mice were used in the TnSeq screen, and both male and female mice were used in the aerosol studies.

Mtb strains

All *Mtb* strains were cultured in Middlebrook 7H9 medium supplemented with oleic acid–albumin–dextrose catalase (OADC), 0.2% glycerol, and 0.05% Tween 80 to log-phase with shaking (200 rpm) at 37°C. Hygromycin (50 µg/mL) and kanamycin (20 µg/mL) were added when necessary. Prior to all *in vivo* infections, cultures were washed, resuspended in phosphate-buffered saline (PBS) containing 0.05% Tween 80 (hereafter PBS-T), and sonicated before diluting to desired concentration.

Mouse infections

For aerosol infections, B6 and D2 were infected with ~50 colony forming units (CFUs) of *Mtb* H37Rv YFP via aerosol inhalation

(Glas-Col) for 6 and 12 weeks. At each timepoint, male and female mice were euthanized in accordance with approved IACUC protocols, and lung and spleen were harvested into PBS-T and homogenized. CFU was quantified by dilution plating onto Middlebrook 7H10 agar supplemented with OADC, 0.2% glycerol, 50 mg/mL carbenicillin, 10 mg/mL amphotericin B, 25 mg/mL polymyxin B, and 20 mg/mL trimethoprim. One lung lobe per mouse was collected into 10% neutral-buffered formalin for hematoxylin and eosin (H&E) staining by the Duke University Research Immunohistology Laboratory.

For TnSeq experiments, 1×10^6 CFU of saturated *Himar1* Tn mutants (Sassetti et al. 2003) was delivered via intravenous tail vein injection, an infection route that enables consistent delivery of the complete TnSeq library to each mouse. Mice were infected in a single batch. At 4-week postinfection, mice were euthanized, and spleens and lungs were harvested and then homogenized in a FastPrep-24 (MP Biomedicals). CFU was quantified by dilution plating on 7H10 agar with 20 μ g/mL kanamycin. For library recovery, $\sim 1 \times 10^6$ CFU per mouse was plated on 7H10 agar with 20 μ g/mL kanamycin. After 3 weeks of growth, colonies were harvested by scraping, and genomic DNA was extracted. The relative abundance of each Tn mutant was estimated as described in Long et al. (2015).

Microscopy and damage quantification

H&E-stained lung sections were imaged in bright field at 2 \times magnification on the Keyence BZ-X800. Images were processed identically within FIJI software (v2.3.0/1.53p) for image clarity. To quantify damage, an artificial neural network-based damage identification model was developed within QuPath (v0.3.2) (Bankhead et al. 2017), as described in Wilburn et al. (2023). Eight images (1 image per experimental group), containing a total of 68 unaffected and 65 affected manual annotations, were utilized for the single purpose of training a pixel classifier, and the remaining 3 images per group were quantified using the model. To prevent bias, lung sections presented in this report were quantified as the closest to the mean damage of each experimental group.

TnSeq analysis

TnSeq libraries were prepared and Tn insertion counts were estimated as previously described (Smith et al. 2022). The H37Rv genome annotation used is NCBI Reference Sequence NC_018143.2. In total, 40 independent Tn libraries were sequenced after recovery from the 19 BXD genotypes and the 2 parent strains, B6 and D2. Two independent Tn libraries were placed under selection within each host genotype. However, in the cases of 2 BXD genotypes, BXD48a and BXD51, only 1 library could be sufficiently recovered for sequencing. Within TRANSIT (DeJesus et al. 2015), beta-geometric correction was used to normalize insertion mutant counts across all libraries, and pseudocounts were implemented. Insertion counts were totaled for each *Mtb* gene, and counts were averaged between individual replicate libraries per mouse. Per gene mean values were compared with the grand mean and then \log_2 transformed.

To control for multiple hypotheses genome wide, 10,000 permutation tests were performed to generate a resampling distribution used to derive the significance of Tn mutant selection between in vitro and in vivo conditions. An *Mtb* gene was considered “essential” within a host if mutants lacking that gene experienced a \log_2 fold change (LFC) value of -0.5 or lower postselection in that host. To identify *Mtb* genes whose fitness was sufficiently variable to enable QTL mapping, the dynamic range in LFC across

the parental and BXD strains for any given bacterial gene had to be at least 0.63. This value is the minimum LFC range of a mutant that was significantly selected in at least 1 host genotype after resampling ($Q < 0.05$). Mutants used for QTL mapping also needed to be sufficiently represented in the library, which was accomplished by requiring 4 or more TA Tn insertion sites to be detected for each bacterial gene. Further, each gene included needed to be significantly selected in at least 1 host genotype before resampling ($P < 0.05$).

Genotyping and QTL mapping

Previously published BXD genotypes containing 7,321 total markers were leveraged for QTL mapping (Wang et al. 2016). Markers that were not diagnostic of genetic differences between B6 and D2 were filtered out resulting in a total of 7,314 markers. Genotype data and phenotype data, including lung and spleen burden and TnSeq mutant fitness profiles, were imported into R statistical software (version 4.1.1) and formatted for QTL mapping within R/qtl2 (version 0.28) (Broman et al. 2019). The leave-one-chromosome-out (LOCO) approach was leveraged to estimate kinship between strains as a covariate for QTL mapping. Because all screened mice were male and infected in 1 batch, no other covariates were used for the initial mapping. Logarithm of odds (LOD) scores were calculated using the linear mixed model in R/qtl2 to establish phenotypic associations with marker loci across the host genome. Trait-wise significance thresholds for QTL were established by 10,000 permutation tests. The significance of gene class overrepresentation among the mapped QTL in comparison with class representation in the whole *Mtb* genome was established using Fisher’s exact test. Chromosomal ideograms of QTL across the mouse genome were generated using R/karyoploteR (Gel and Serra 2017).

Results

B6 and D2 mouse strains experience distinct chronic disease profiles after aerosol infection

B6 and D2, the parent strains of the BXD panel, have divergent responses to *Mtb* infection (Chackerian and Behar 2003). Canonically considered *Mtb* resistant, the T_H1 -skewed adaptive immune response initiated by B6 mice is capable of restraining *Mtb* growth after 25 days, resulting in a mean survival time of ~ 230 days (Mitsos et al. 2000). D2 mice control early infection with *Mtb* equally as well as B6 mice until ~ 25 days. However, D2 mice experience low IFN- γ -producing T-cell influx to the lung when adaptive immunity should begin, at which point B6 and D2 begin to diverge in bacterial growth restriction (Fig. 1a and b) and lung damage (Fig. 1c–e) (Marquis et al. 2008). D2 macrophages become foamy and dysfunctional, making it difficult to contain and control bacterial growth in the lung (Chackerian and Behar 2003). By 12-week post-aerosol infection, D2 lungs exhibit hyperinflammatory and fibrotic responses leading to overt damage (Fig. 1f–h) and an ultimate mean survival time of ~ 110 days (Medina and North 1998; Keller et al. 2006). In addition to genotype-specific differences between these BXD parent strains, we also observe a sex effect independent of genotype, demonstrated by higher susceptibility of males of both genotypes (Supplementary Fig. 1a and b) (Tsuyuguchi et al. 2001; Dibbern et al. 2017). Further, we find that in contrast to burden, all groups reduce lung damage by week 12 except for D2 males (Supplementary Fig. 1c–g).

Table 1. Genome-wide significant QTL ($P \leq 0.05$).

hpQTL	Trait	Gene	Chr	Position (Mb)	LOD	Start (Mb)	End (Mb)	P-value
hp001	Rv0913c	Rv0913c	1	22.773	3.52	5.022	73.222	2.24E-02
hp002	Rv0778	cyp126	1	121.148	3.98	115.474	126.340	1.67E-02
hp003	Rv2468c	Rv2468c	1	152.965	4.54	147.055	166.299	2.33E-02
hp004	Rv1260	Rv1260	1	155.701	4.29	143.711	160.273	3.94E-02
hp005	Rv0103c	ctpB	1	189.196	3.09	3.010	195.308	4.60E-02
hp006	Rv1525	wbbL2	2	10.808	3.56	5.653	14.395	3.32E-02
hp007	Rv3400	Rv3400	2	24.076	4.12	17.898	42.375	8.38E-03
hp008	Rv0625c	Rv0625c	2	45.533	3.37	25.645	161.538	5.01E-02
hp009	Rv0599c	vapB27	2	46.212	3.74	42.557	54.975	7.86E-03
hp010	Rv3294c	Rv3294c	2	138.850	3.56	136.128	158.741	3.73E-02
hp011	Rv3565	aspB	2	159.316	3.88	42.557	174.820	1.47E-02
hp012	Rv2546	vapC18	2	169.114	4.90	168.446	169.887	1.80E-03
hp013	Rv1965	yrbE3B	2	170.362	3.50	15.435	180.835	1.43E-02
hp014	Rv1367c	Rv1367c	2	173.385	3.77	46.133	176.951	1.85E-02
hp015	Rv1290A	Rv1290A	2	181.009	2.96	14.698	181.991	2.36E-02
hp016	Rv3047c	Rv3047c	3	41.212	3.92	39.644	51.561	4.49E-02
hp017	Rv0101	nrp	3	57.642	3.69	42.035	61.219	6.19E-03
hp018	Rv2051c	ppm1	3	62.392	4.25	61.219	70.185	1.94E-02
hp019	Rv1935c	echA13	3	129.496	4.23	10.997	157.792	4.20E-02
hp020	Rv1735c	Rv1735c	3	129.508	3.64	117.504	157.140	4.20E-02
hp021	Rv2602	vapC41	3	132.174	4.01	129.509	138.245	1.12E-02
hp022	Rv0773c	ggtA	3	146.239	4.56	143.570	156.034	2.75E-02
hp023	Rv0077c	Rv0077c	4	144.942	4.31	144.913	148.864	1.04E-02
hp024	Rv0588	yrbE2B	4	153.793	3.76	54.951	155.498	3.23E-02
hp025	Rv0093c	Rv0093c	5	28.411	3.05	19.137	132.038	2.29E-02
hp026	Rv0768	aldA	5	43.085	4.19	29.537	52.408	3.09E-03
hp027	Rv2957	Rv2957	5	117.550	3.27	3.143	139.501	3.26E-02
hp028	Rv3848	Rv3848	5	136.015	5.69	133.911	138.657	1.55E-02
hp029	Rv0359	rip2	6	76.492	4.18	75.970	82.500	5.63E-03
hp030	Rv0955	perM	6	82.500	4.80	80.286	83.092	1.29E-02
hp031	Rv2140c	TB18.6	6	84.277	4.62	80.286	85.650	2.28E-02
hp032	Rv3849	espR	6	84.869	6.77	83.684	85.566	6.23E-03
hp033	Rv0127	mak	6	85.650	7.83	84.277	88.578	7.04E-03
hp034	Rv1056	Rv1056	6	93.600	4.10	89.371	95.127	4.40E-02
hp035	Rv2671	ribD	6	148.818	3.53	12.797	149.266	1.60E-02
hp036	Rv3711c	dnaQ	7	27.300	3.31	24.735	145.321	3.80E-02
hp037	Rv3299c	atsB	7	36.692	3.01	3.078	144.446	5.34E-02
hp038	Rv0065	vapC1	7	37.008	3.41	3.078	144.445	4.72E-02
hp039	Rv1530	adh	7	37.008	3.22	3.078	145.321	3.74E-02
hp040	Rv2482c	plsB2	7	37.008	3.55	24.619	144.445	2.35E-02
hp041	Rv0026	Rv0026	7	37.760	3.34	24.764	128.756	2.82E-02
hp042	Rv1913	Rv1913	7	39.822	3.63	30.689	145.321	2.96E-03
hp043	Rv1236	sugA	7	40.289	3.13	24.822	140.297	5.28E-03
hp044	Rv1528c	papA4	7	40.289	4.97	40.226	46.357	2.11E-02
hp045	Rv1542c	glnB	7	40.289	3.71	3.078	145.321	5.77E-02
hp046	Rv1992c	ctpG	7	40.289	3.24	6.015	145.321	3.20E-02
hp047	Rv3702c	Rv3702c	7	40.289	4.55	24.793	140.297	2.75E-02
hp048	Rv2388c	hemN	7	84.014	3.39	39.894	127.779	2.54E-02
hp049	Rv0852	fadD16	7	97.465	3.71	83.879	128.058	3.89E-02
hp050	Rv0878c	PPE13	7	97.685	4.10	89.870	127.500	2.49E-02
hp051	Rv0089	Rv0089	7	116.709	4.08	98.124	127.989	3.64E-02
hp052	Rv1912c	fadB5	7	134.484	4.49	51.060	134.831	1.14E-02
hp053	Rv3868	eccA1	7	134.598	3.80	17.136	144.445	4.40E-02
hp054	Rv2637	dedA	7	140.297	4.63	138.137	140.586	1.63E-02
hp055	Rv0271c	fadE6	7	140.586	5.74	140.586	143.684	3.53E-02
hp056	Rv1984c	cfp21	7	140.586	3.44	24.938	144.445	1.68E-02
hp057	Rv1104	Rv1104	7	143.809	3.29	24.735	145.321	2.49E-02
hp058	Rv3179	Rv3179	7	143.809	3.78	140.586	144.445	5.34E-02
hp059	Rv1748	Rv1748	7	144.184	5.42	37.760	144.446	7.25E-03
hp060	Rv3288c	usfY	7	144.184	3.97	30.593	145.321	3.46E-02
hp061	Rv3698	Rv3698	7	144.309	3.41	12.026	145.321	3.60E-02
hp062	Rv1245c	Rv1245c	7	144.446	3.97	40.289	145.321	2.62E-03
hp063	Rv2092c	helY	7	144.687	5.34	144.533	145.321	2.50E-02
hp064	Rv0458	Rv0458	7	145.321	3.92	144.448	145.321	2.93E-02
hp065	Rv0538	Rv0538	8	11.279	4.57	11.279	13.680	1.47E-02
hp066	Rv2066	cobI	8	14.819	4.31	12.880	15.343	4.00E-02
hp067	Rv1039c	PPE15	8	16.687	4.55	13.680	16.946	1.31E-02
hp068	Rv3855	ethR	8	87.897	4.62	87.219	89.367	1.44E-02
hp069	Rv2161c	Rv2161c	8	113.708	4.09	109.407	128.590	5.93E-03
hp070	Rv2384	mbtA	9	29.939	4.02	4.359	107.777	3.24E-02
hp071	Rv1954c	Rv1954c	9	34.618	4.70	29.941	58.854	2.24E-02

(continued)

Table 1. (continued)

hpQTL	Trait	Gene	Chr	Position (Mb)	LOD	Start (Mb)	End (Mb)	P-value
hp072	Rv3899c	Rv3899c	9	94.905	4.16	87.462	101.324	3.61E-02
hp073	Rv0950c	Rv0950c	9	101.365	3.92	4.359	106.019	2.64E-02
hp074	Rv0538	Rv0538	9	103.686	4.05	80.003	106.019	4.83E-02
hp075	Rv3087	Rv3087	10	5.031	4.52	3.181	9.092	3.38E-02
hp076	Rv2529	Rv2529	10	9.413	4.33	3.181	11.991	9.41E-03
hp077	Rv1450c	PE_PGRS27	10	19.780	4.04	13.853	21.509	3.92E-02
hp078	Rv1507c	Rv1507c	10	55.483	4.97	55.356	59.293	1.73E-02
hp079	Rv1366	Rv1366	10	64.014	3.25	57.276	72.506	2.31E-02
hp080	Rv1552	frdA	10	96.749	3.97	95.639	103.562	3.45E-02
hp081	Rv1753c	PPE24	10	109.165	3.52	13.688	127.327	1.75E-02
hp082	Rv2261c	Rv2261c	10	111.074	4.75	110.998	126.607	1.16E-02
hp083	Rv0044c	Rv0044c	10	115.897	3.42	3.181	127.808	6.21E-02
hp084	Rv2326c	Rv2326c	10	120.610	4.29	116.134	121.419	1.26E-02
hp085	Rv0769	Rv0769	10	126.740	4.12	123.469	126.808	2.36E-02
hp086	Rv2993c	Rv2993c	11	11.620	4.35	8.814	15.810	3.65E-02
hp087	Rv3759c	proX	11	21.073	4.70	19.834	34.416	4.71E-02
hp088	Rv0086	hycQ	11	24.850	3.37	18.284	35.583	1.22E-02
hp089	Rv3841	bfrB	11	47.049	3.28	35.903	80.586	1.67E-02
hp090	Rv0126	treS	11	65.541	3.56	64.895	81.165	3.85E-02
hp091	Rv2116	lppK	11	73.243	4.46	73.194	81.165	1.67E-02
hp092	Rv0765c	Rv0765c	11	106.661	4.70	106.040	107.457	2.58E-02
hp093	Rv1850	ureC	11	107.742	4.25	107.422	110.228	1.61E-02
hp094	Rv2802c	Rv2802c	11	107.742	4.87	107.133	109.751	9.90E-03
hp095	Rv1452c	PE_PGRS28	11	107.964	3.29	103.707	111.341	4.10E-02
hp096	Rv0599c	vapB27	11	113.557	3.58	110.546	118.682	1.76E-02
hp097	Rv1969	mce3D	12	46.628	4.14	27.823	52.908	4.09E-02
hp098	Rv3554	fdxB	12	71.873	3.22	58.970	114.110	4.64E-02
hp099	Rv1448c	tal	12	79.511	4.90	76.378	86.909	1.38E-02
hp100	Rv2922c	smc	12	105.499	4.57	104.281	107.191	3.70E-02
hp101	Rv2268c	cyp128	12	107.369	3.62	5.594	107.848	3.59E-02
hp102	Rv1775	Rv1775	12	108.105	3.71	107.369	108.898	3.33E-02
hp103	Rv3164c	moxR3	12	114.166	4.13	111.180	116.685	3.60E-02
hp104	Rv2019	Rv2019	13	6.304	3.82	4.432	115.537	4.09E-02
hp105	Rv2379c	mbtF	13	18.035	3.66	4.432	45.238	3.69E-02
hp106	Rv0066c	icd2	13	39.372	4.40	34.110	39.515	1.60E-02
hp107	Rv1250	Rv1250	13	50.000	3.82	45.902	54.614	2.04E-02
hp108	Rv2282c	Rv2282c	13	54.862	3.93	54.843	72.958	3.30E-02
hp109	Rv2074	Rv2074	13	63.704	5.16	61.332	71.002	6.80E-03
hp110	Rv0552	Rv0552	13	67.497	5.30	63.792	67.497	3.26E-02
hp111	Rv1009	rpfB	13	75.762	4.75	72.969	84.394	2.23E-02
hp112	Rv0223c	Rv0223c	14	73.646	4.05	73.198	79.374	2.12E-02
hp113	Rv3063	cstA	14	79.403	3.63	61.301	118.199	1.55E-02
hp114	Rv1525	wbbL2	15	27.520	3.75	11.145	102.347	1.79E-02
hp115	Rv3013	Rv3013	15	28.265	3.98	12.673	28.312	1.65E-02
hp116	Rv3354	Rv3354	15	28.312	2.83	9.237	102.347	4.35E-02
hp117	Rv1664	pks9	15	32.262	2.86	7.168	72.449	3.57E-02
hp118	Rv1366	Rv1366	15	64.513	3.43	42.380	84.022	9.72E-03
hp119	Rv3765c	trcX	15	74.502	4.19	69.446	75.001	6.29E-03
hp120	Rv0454	Rv0454	15	75.750	4.13	75.001	82.088	1.87E-02
hp121	Rv2315c	Rv2315c	15	84.037	3.37	7.814	102.354	4.37E-02
hp122	Rv0242c	fabG4	15	88.975	5.10	86.329	90.948	5.91E-03
hp123	Rv0959	Rv0959	15	91.863	4.47	90.948	93.408	4.13E-02
hp124	Rv1961	Rv1961	15	95.169	4.46	94.529	96.191	5.17E-02
hp125	Rv0043c	Rv0043c	16	44.080	3.98	36.743	44.128	2.51E-02
hp126	Rv1400c	lipI	16	78.088	3.52	75.905	85.637	3.92E-02
hp127	Rv1552	frdA	16	92.557	3.95	74.904	96.503	3.60E-02
hp128	Rv3058c	Rv3058c	17	55.278	4.32	6.007	76.812	2.40E-04
hp129	Rv1507A	Rv1507A	17	85.527	4.44	80.477	87.364	6.91E-03
hp130	Rv3776	Rv3776	17	94.132	4.63	91.701	94.411	1.16E-02
hp131	Rv3495c	lprN	18	57.375	4.87	57.348	58.270	3.71E-02
hp132	Rv1084	Rv1084	19	10.714	4.90	3.337	14.209	2.73E-02
hp133	Rv2378c	mbtG	19	23.841	3.02	21.915	27.750	3.72E-02
hp134	Rv0056	rplI	19	39.978	4.00	38.651	42.427	3.83E-02
hp135	Rv3540c	ltp2	X	101.772	4.43	80.056	115.391	3.14E-03
hp136	Rv0694	lldD1	X	115.365	3.91	102.867	167.395	3.70E-02
hp137	Rv1791	PE19	X	119.825	4.38	115.497	128.620	2.38E-02
hp138	Rv2733c	Rv2733c	X	133.591	3.40	115.471	138.012	3.90E-02
hp139	Rv0182c	sigG	X	136.470	4.68	136.304	138.007	1.61E-02
hp140	Rv1413	Rv1413	X	138.012	4.90	135.586	139.435	5.84E-03

“hpQTL” is the name for each host-pathogen QTL of genome-wide significance ($P \leq 0.05$). “Trait” refers to the Rv number of the bacterial mutant whose fitness profile identified the hpQTL in the host genome. “Gene” is the bacterial gene annotation of the mutant if one is available on Mycobrowser. “Chr” is the host chromosome on which the QTL is located, and “Position (Mb)” is the position of the QTL in megabases. “LOD” is the maximum association score between the mutant fitness profile and the host locus. “Start (Mb)” and “End (Mb)” are the lower and upper boundaries of the Bayesian 95% confidence interval, as calculated by R/qtl2. “P-value” denotes the significance of the association between the bacterial mutant fitness and the host genetic locus.

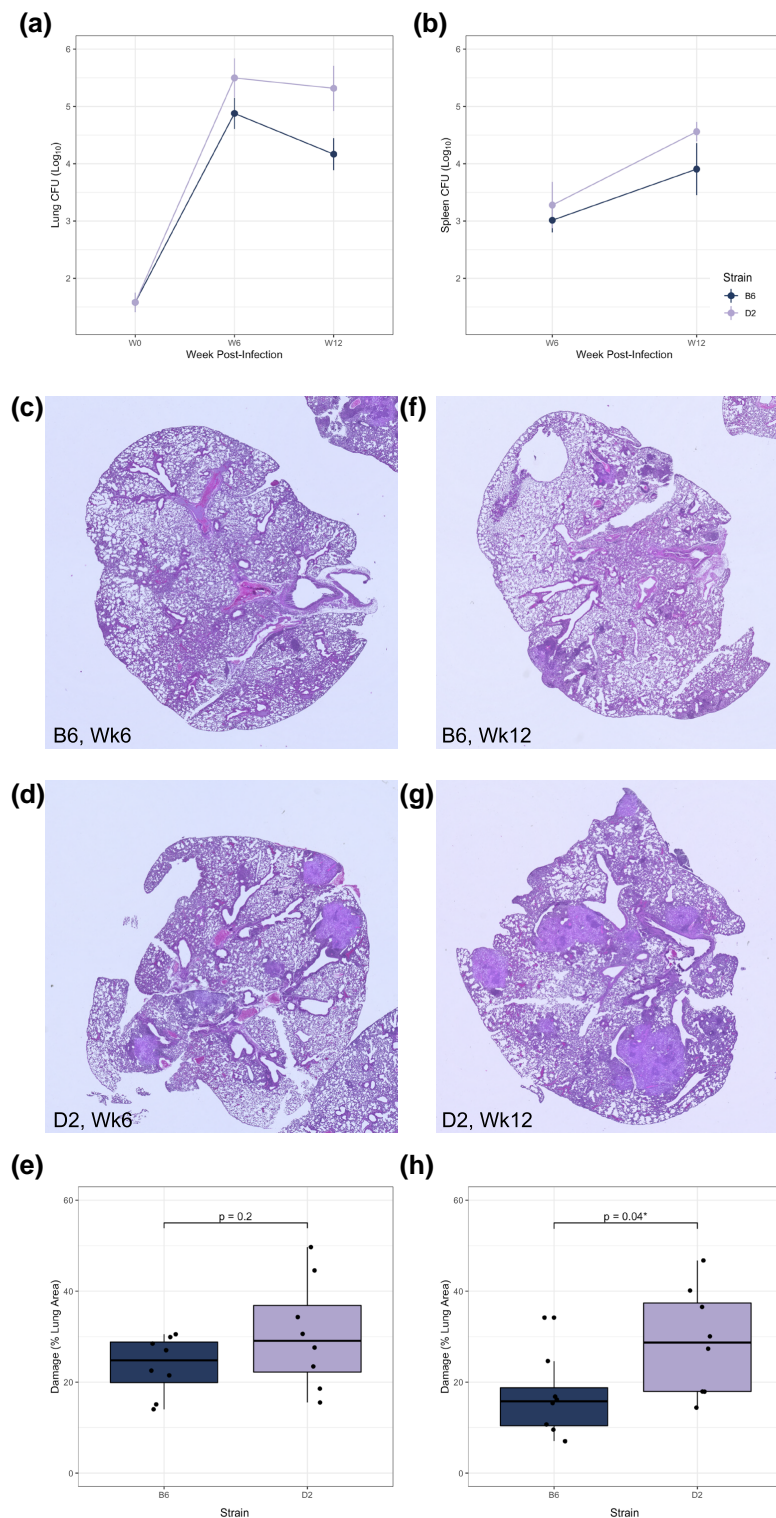


Fig. 1. B6 and D2 are phenodeviant in TB susceptibility. a) Log₁₀ transformed CFUs recovered from lung tissue throughout the course of the aerosol infection (with an infectious dose of ~40 CFU of *Mtb* H37Rv). b) Log₁₀ transformed CFU recovered from spleen throughout infection. c and d) Male B6 and D2 H&E-stained lung sections taken 6-week postinfection, 2× magnification, representative of $n = 4$ per strain. Corresponding female sections can be found in [Supplementary Fig. 1c and d](#). e) Lung damage was quantified by QuPath v0.3.2 using an artificial neural network-based damage identification model and reported as a percent of total lung section area ($n = 4$ per genotype and sex). Lung damage is not significantly different between B6 and D2 at 6-week postinfection by unpaired Student's *t*-test. f and g) Male B6 and D2 H&E-stained lung sections taken 12-week postinfection, 2× magnification, representative of $n = 4$ per strain. Corresponding female sections can be found in [Supplementary Fig. 1e and f](#). h) Lung damage, quantified as in e), is significantly different between B6 and D2 at 12-week postinfection by unpaired Student's *t*-test.

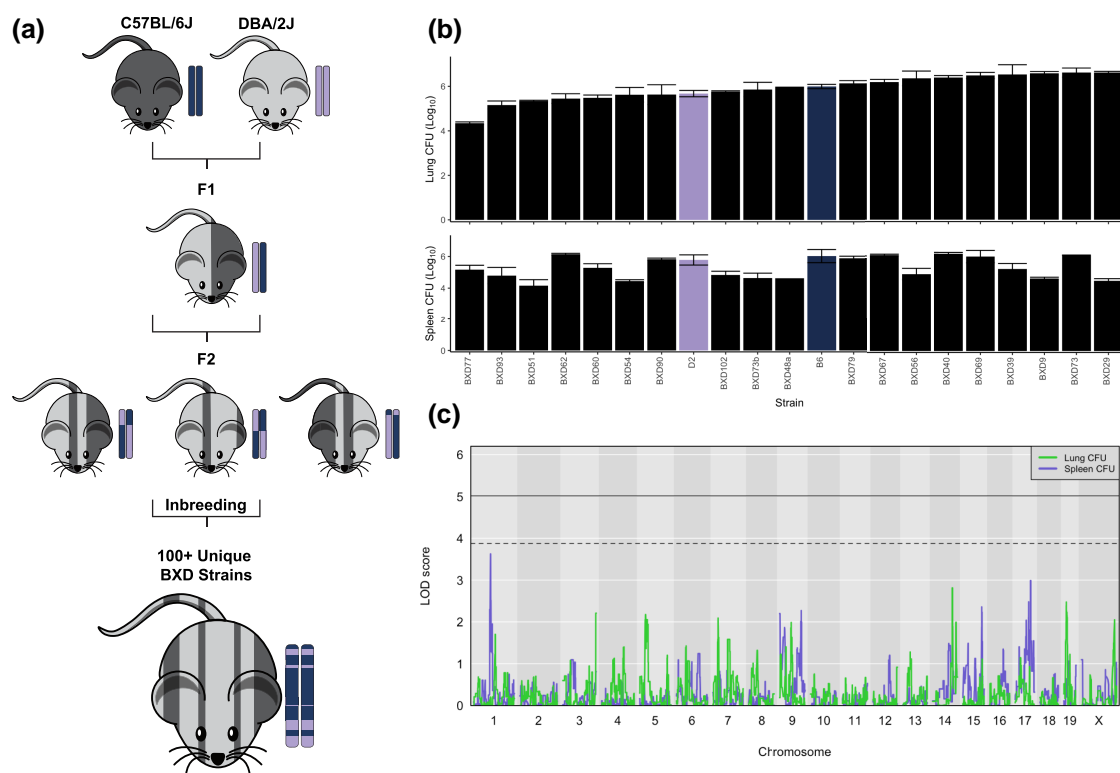


Fig. 2. BXD panel exhibits natural genetic variation, but classical clinical traits cannot map genome-wide QTL at 4-week postinfection. a) The BXD panel is a biparental recombinant inbred panel bred from C57BL/6J (B6) and DBA/2J (D2) and composed of over 100 mosaic strains. b) Lung and spleen burden by host genotype ($n = 1-5$ per genotype). c) QTL mapping of lung and spleen burden traits across the BXD cohort. Solid threshold represents a genome-wide significance of $P = 0.05$. Dashed threshold represents $P = 0.20$. Thresholds were calculated from 10,000 permutation tests.

Early clinical macrophenotypes fail to distinguish parental disease outcomes

With over 100 still extant recombinant inbred strains bred from B6 and D2 parental strains, the BXD family is a well-suited mammalian resource to map the phenotypic diversion during *Mtb* infection (Fig. 2a). To study early disease traits and bacterial endophenotypes that predict differences in TB disease outcomes during chronic infection stages, we intravenously infected the parental B6 and D2 lines and 19 BXD genotypes with a saturated TnSeq library of Tn mutants. The library contains knockouts of every *Mtb* gene that is not required in vitro, which collectively produces an infection similar to the wild-type infection strain (Sassetti and Rubin 2003; Smith et al. 2022). At 4-week postinfection, the parental B6 and D2 strains demonstrated approximately the same bacterial burden in lung and spleen (Fig. 2b). Moreover, BXD strains did not exhibit sufficiently wide variation in bacterial burden at 4-week postinfection to precisely map the genetic cause of such a complex trait (Fig. 2c; Supplementary Fig. 2a and b). Compound macrophenotypes, such as CFUs in organ homogenate, are highly polygenic, making it difficult to identify causal genes even with large panels of mice (Lavebratt et al. 1999; Mitsos et al. 2000; Mitsos et al. 2003; Yan et al. 2006).

Tn mutant fitness endophenotypes report on the host immunological microenvironment

To interrogate the biological mechanisms that predict immunodivergent outcomes, we quantified the abundance of each bacterial mutant both before infection with the TnSeq library and after recovery from mouse organs of each genotype, using the LFC in

abundance as a quantification of relative mutant fitness in each condition. This LFC fitness value serves as a precise reporter of the host microenvironment and can be used to map host loci that impact *Mtb* mutant fitness (Fig. 3a).

We identified 3 main classes of bacterial mutants, which are highlighted in Fig. 3b. The first of these classes includes canonical *Mtb* genes that are essential for in vivo survival, exemplified by *bioA*. These genes remain essential for *Mtb* survival in both parental backgrounds and across the BXD strains (Fig. 3b; “essential” profile in red). A second, “differentially required” set of mutants is exemplified in this figure by *mbtG*; the loss of *mbtG* did not significantly reduce bacterial fitness within B6 mice, but *mbtG* mutants experienced an extreme fitness reduction within D2 mice (Fig. 3b; “differentially required” profile in yellow). Further, there is a spectrum of *mbtG* essentiality across the family, indicating that some variable host factor could modify the fitness of this mutant. In addition to in vivo essential and differentially essential *Mtb* genes, a third class of *Mtb* genes proved to be broadly dispensable for *Mtb* survival in these hosts, imparting an adaptive benefit for *Mtb* when the gene was disturbed, represented here by *glpK* (Fig. 3b; “nonessential” profile in green).

When surveying the complete set of *Mtb* Tn mutants, we find that in comparison with B6 infection, *Mtb* requires almost twice as many genes to survive within D2 mice (Fig. 3c). This finding suggests that there are detectable immunological pressures being exerted on *Mtb* within the highly inflammatory D2 line prior to the divergence in B6 and D2 bacterial control and disease outcome. This result replicates findings from previous work in which a greater number of *Mtb* genes were essential in T-cell-deficient and IFN- γ -deficient mice than in B6 (Mishra et al. 2017; Smith

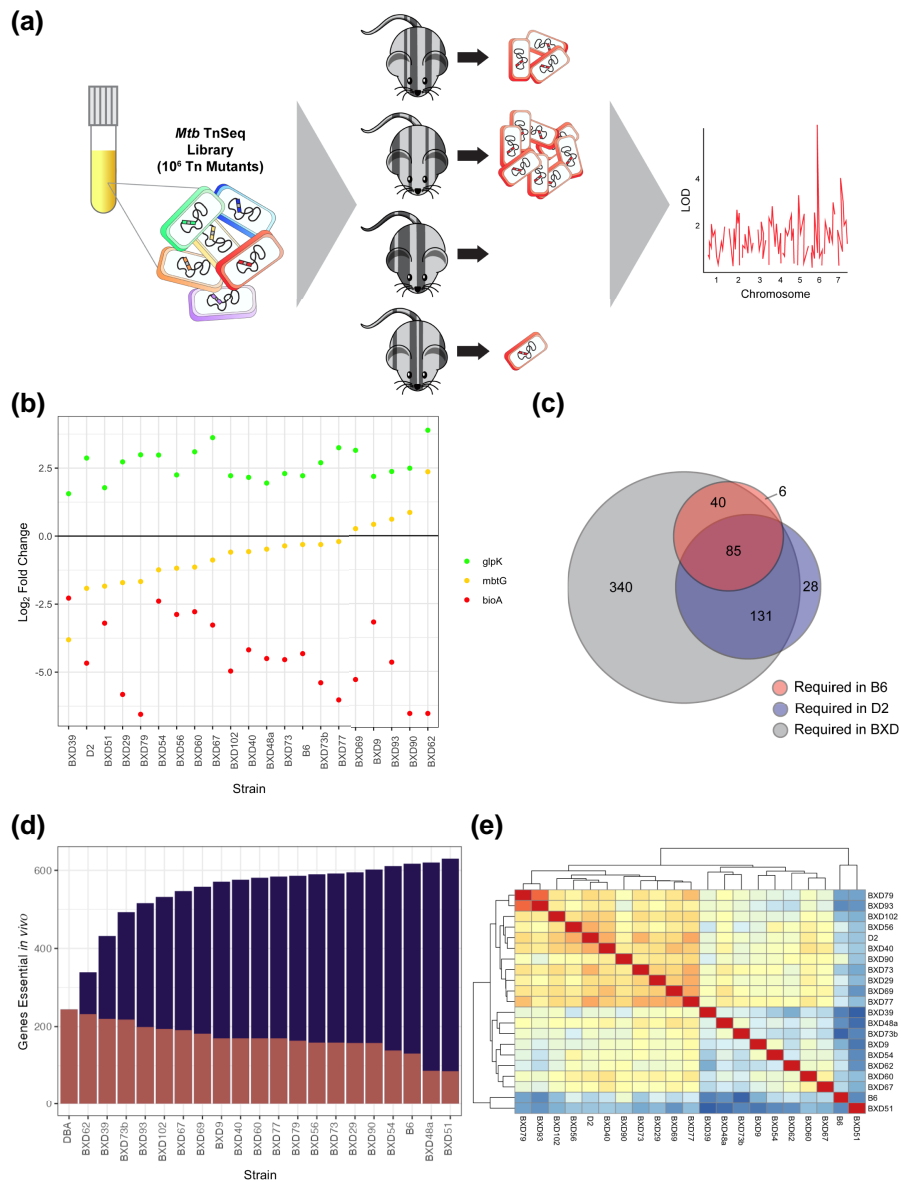


Fig. 3. TnSeq mutant fitness is a sensitive reporter of the factors that drive disease outcome. a) Each BXD and parental strain was infected with 10^6 CFU of *Mtb* TnSeq library, representative of $\sim 10^5$ independent *Mtb* Tn mutants. For each mutant, the change between the initial abundance in the TnSeq library and the abundance recovered after 4 weeks of in vivo infection was compared with calculate a quantitative fitness metric (LFC), which could be used downstream for QTL mapping. b) Example plot showing an essential *Mtb* gene (*bioA*), a conditionally essential gene (*mbtG*), and a nonessential gene (*glpK*) across the BXD panel. c) A Venn diagram depicting the overlap of essential genes between B6, D2, and the screened BXD strains. *Mtb* genes were deemed essential if the Tn mutant experienced a 1-fold reduction (LFC < -0.5) within a given host strain and were significantly different from in vitro conditions ($P < 0.05$) in at least 1 host strain across the panel. d) The number of *Mtb* genes essential (LFC < -0.5) for growth or survival in each diverse mouse strain across the panel ($P < 0.05$). Salmon (smallest circle) indicates the mutants uniquely required for each host additional strain, and purple (medium circle) shows the cumulative requirement as each new host strain is added. e) A heatmap depicting the total correlation of the library-wide bacterial fitness within each screened host strain.

et al. 2022). We additionally find that the BXD panel, while recapitulating most B6- and D2-essential genes, reveals additional subsets of genes that are only required within specific BXD strains (Fig. 3d), highlighting the power of this genetically diverse panel to offer novel insights into the biology controlling TB outcome.

This spectrum of *Mtb* gene essentiality is further confirmed when we examine the correlation of the complete TnSeq library fitness between hosts (Fig. 3e). Interestingly, the majority of the strains hierarchically clustered closer to D2 than to B6, indicating that they could be inheriting factors from D2 that put greater immunological pressure on *Mtb*.

Tn mutant fitness profiles map *hp*QTL

We aimed to use these bacterial mutants as sensitive reporters of host immunological pressures within the early phase of infection at the onset of adaptive immunity. Using the LFC values collected for each Tn mutant population in the TnSeq library as quantitative endophenotypes (Supplementary Table 1), we conducted QTL mapping. After removing nondynamic genes for quality control (Supplementary Fig. 3a; *Mtb* genes that are not sufficiently varying in essentiality across the screened BXD genotypes), we found 140 genome-wide significant QTL, defined as $P \leq 0.05$ after 10,000 permutations (Table 1). Of these QTL, 33 of the loci had a P -value of ≤ 0.01 (Fig. 4a).

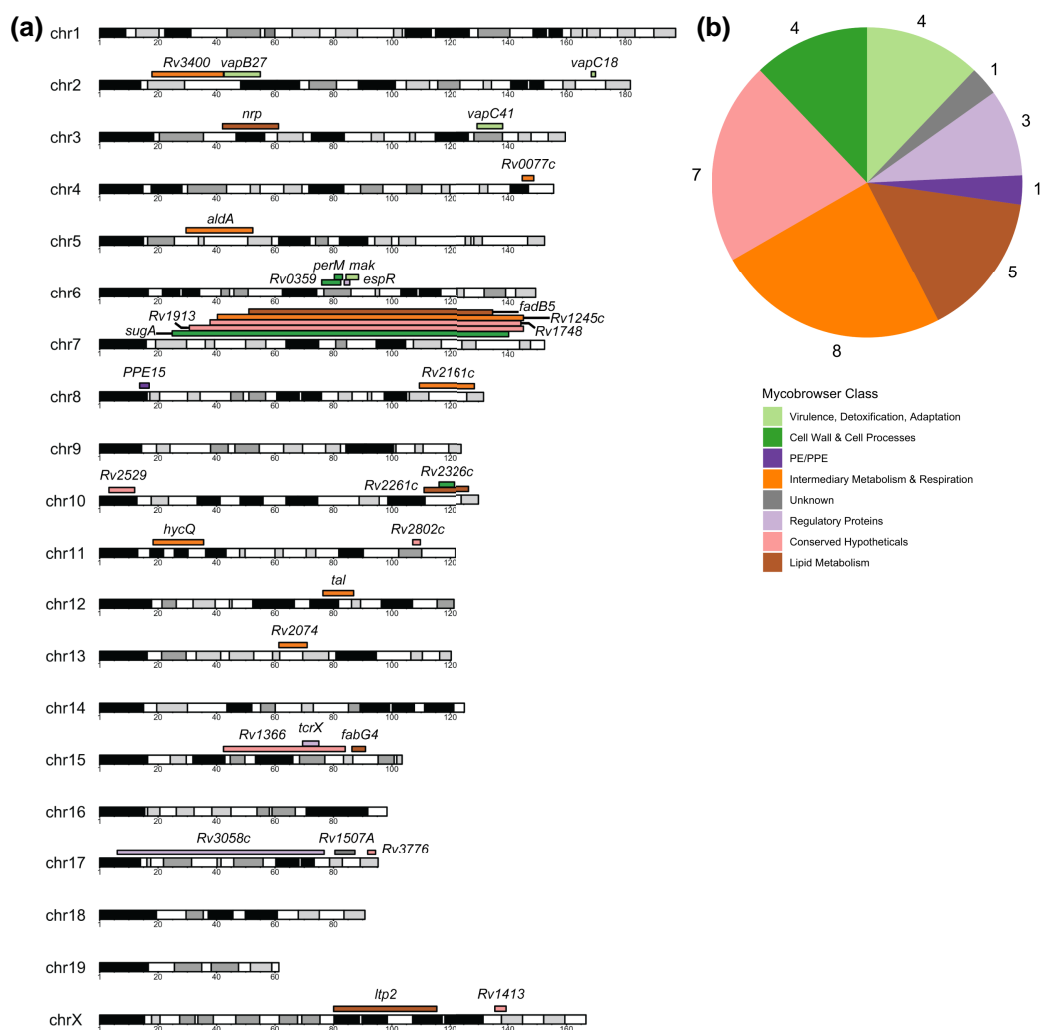


Fig. 4. TnSeq mutant fitness endophenotypes map genome-wide significant QTL. a) The QTL that reach a significance threshold of $P \leq 0.01$. The color of the QTL corresponds to the Mycobrowser class of the gene absent from the Tn mutant that mapped the QTL. The width of each segment corresponds to the size of the 95% Bayesian confidence interval of the QTL as calculated by R/qt12. b) A pie chart tabulating the Mycobrowser classes of the mutants that mapped highly significant QTL ($P \leq 0.01$), beyond the genome-wide significance threshold of $P \leq 0.05$.

Among the most significant QTL mapped in the screen, certain gene classes were marginally overrepresented in comparison with the genome-wide abundance of that class. Tn mutants lacking genes that encode known virulence factors made up 12.1% of the top hits in this screen despite only representing 5.8% of the *Mtb* genome ($P = 0.1$) (Fig. 4b) (Kapopoulou et al. 2011). Further, lipid metabolism genes made up 15.2% of the bacterial mutants mapping highly significant QTL while representing 6.6% of the *Mtb* genome ($P = 0.07$). Certain gene classes were abundant in approximately equal proportions in the mapped QTL as they were genome wide, such as metabolism and respiration genes (22.8% among top QTL vs 24.2% genome-wide prevalence) and genes encoding PPE/PE proteins (3.0% among top QTL vs 4.1% genome-wide prevalence). Roughly half of *Mtb* protein-coding genes remain unannotated (Whitaker et al. 2020), and 7 of the highly significant ($P \leq 0.01$) bacterial fitness traits mapped by this screen correspond to bacterial genes with no known function. Taking these screen data in combination with previous in vivo TnSeq studies, these data will contribute to deorphaning the *Mtb* genome.

Multiple highly significant QTL map to host chromosome 6 “hotspot”

The nature of this screen enabled the discovery of host loci that impact the fitness of numerous bacterial mutants. Among the QTL mapped in this screen, 5 QTL mapped to chromosome 7 (Fig. 4a), yet the 95% confidence intervals for each QTL span such a large part of the chromosome that identifying a causal host gene would be infeasible without further experimentation. Aside from the chromosome 7 locus, there appears to be a much narrower region on chromosome 6 (75.97–88.58 Mb) strongly linked to the essentiality of 4 bacterial genes: *Rv0127* (*mak*; sometimes annotated as *pep2*), *Rv0359* (*rip2*), *Rv0955* (*perM*), and *Rv3849* (*espR*) (Fig. 5a–e). For each of the mutants lacking these bacterial genes, apart from *perM*, the B6 sequence in this region is associated with lower bacterial mutant fitness (Fig. 5f–i; Supplementary Fig. 4a–h). *Mtb* mutants lacking *perM* experience a fitness cost within genotypes that possess the D haplotype in this region.

This QTL hotspot is mapped by *Mtb* genes that impact metabolism, virulence, and defense. *Mak* catalyzes the ATP-dependent

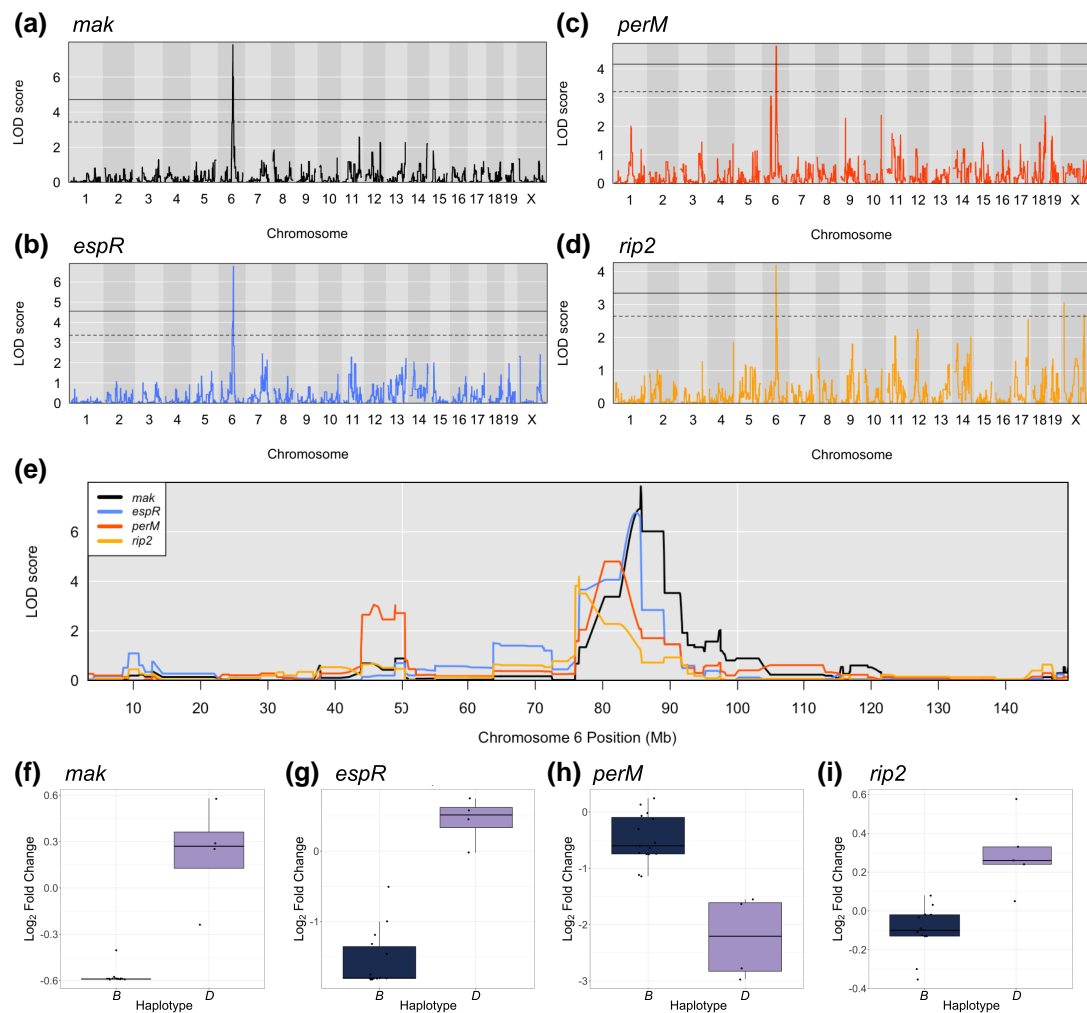


Fig. 5. A chromosome 6 QTL hotspot highlights a genomic region controlling fitness of multiple bacterial mutants. QTL mapping of Tn mutants lacking a) *mak*, b) *espR*, c) *perM*, and d) *rip2*. After 10,000 permutation tests, the solid threshold represents $P = 0.05$, and the dashed threshold represents $P = 0.20$. e) Chromosome 6 mapping overlap of the 4 Tn mutant fitness profiles that identified the QTL hotspot. f–i) Boxplots representing the fitness of Tn mutants lacking *mak*, *espR*, *perM*, and *rip2* within BXD mice with B (navy) or D (lilac) haplotypes at the QTL position. BXD genotypes for which a haplotype state could not be called with 95% confidence were not included.

conversion of maltose into maltose-1-phosphate as a part of the glycogen biosynthesis pathway that supports construction of the protective outer capsule of *Mtb* (Li et al. 2014). Of note, TreS, which converts trehalose to maltose upstream of Mak, also maps to a genome-wide significant QTL on chromosome 11 and has done so in previous *Mtb* infection screens (Smith et al. 2022). *rip2* is a putative zinc metalloprotease located in the *Mtb* cell membrane (Sklar et al. 2010), although very little is known about its function. PerM is an integral membrane protein that is known to play an essential role in enabling bacterial cell division under acid stress in B6 mice (Wang et al. 2019). Here, we report that *perM* mutants are even more severely attenuated within a D2 background, implying that PerM is differentially impacted by host immunological pressures. EspR is a key regulator of the ESX-1 secretion system (Rosenberg et al. 2011; Blasco et al. 2012), which itself plays a well-established role in mycobacterial virulence. Mutants lacking these bacterial genes map significant QTL within the interval of 75.97–88.58 Mb on mouse chromosome 6.

The 95% Bayesian confidence intervals of the QTL mapped by *rip2* and *perM* mutants overlap while the confidence intervals of *mak* and *espR* mutants overlap separately from the *rip2* and *perM* QTL. To test for the independence of these 2 groups of nearby

QTL, we remapped each of the QTL within the chromosome 6 hotspot, iteratively incorporating the haplotype possessed by each BXD strain at each QTL as an additive covariate. Dependent loci will not map if the haplotype of 1 locus is factored into the mapping pipeline as a covariate. Conversely, if a QTL achieves significance by permutation test while the haplotype state at another significant QTL nearby is factored into mapping as a covariate, it suggests that the 2 loci arise from distinct causal genetic factors within the host. These Scan2 independence tests, so named in R/qtl, suggested 2 causal host loci exist within this highly significant QTL hotspot: 1 host factor underlying the *rip2* and *perM* mutant fitness QTL and 1 underlying the *mak* and *espR* QTL (Fig. 6a–d).

To identify host gene candidates that may mediate the survival of these 2 groups of bacterial mutants, we defined a pipeline to select for protein-coding genes for which D haplotype has a unique SNP in comparison with B haplotype (Fig. 7a). We passed each of the genes identified by this pipeline through a list of genetic and clinical criteria using independent but complementary data sets from a variety of *Mtb*-infected mammalian cohorts (Fig. 7b) (Zak et al. 2016; Moreira-Teixeira et al. 2017; Ahmed et al. 2020). From this comparative analysis, we identified a putative host candidate within the region mapped by *mak*: MAX dimerization

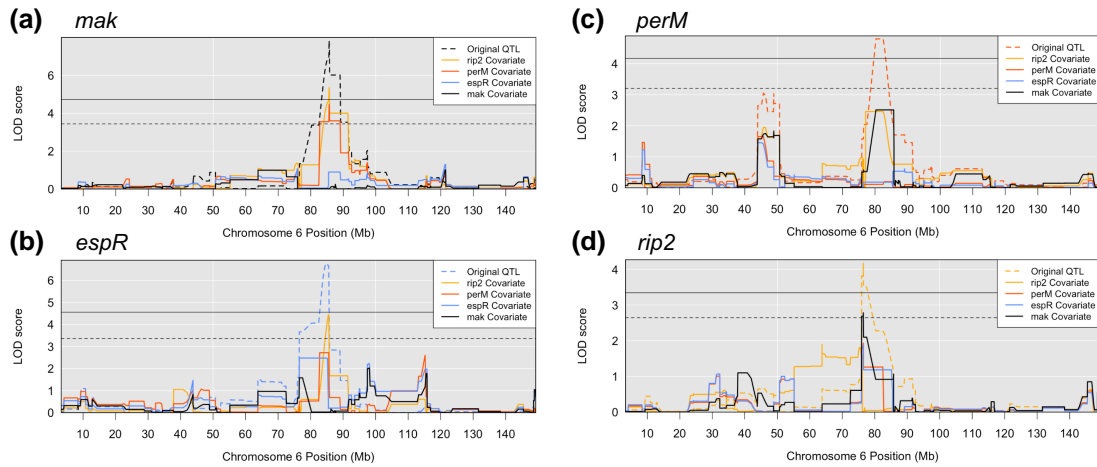


Fig. 6. Independence tests identify 2 putative causal variants. Scan2 independence tests from R/qtl were used to identify whether more than 1 causal host factor may underlie the chromosome 6 hotspot. The QTL mapped by a) *mak*, b) *espR*, c) *perM*, and d) *rip2* Tn mutants were remapped, incorporating the haplotype of the BXD mice at the locus of each other QTL as a covariate in the mapping analysis. The original QTL mapped by each mutant fitness profile are represented as dashed lines. The solid horizontal threshold represents $P = 0.05$ while the dashed horizontal threshold represents $P = 0.20$.

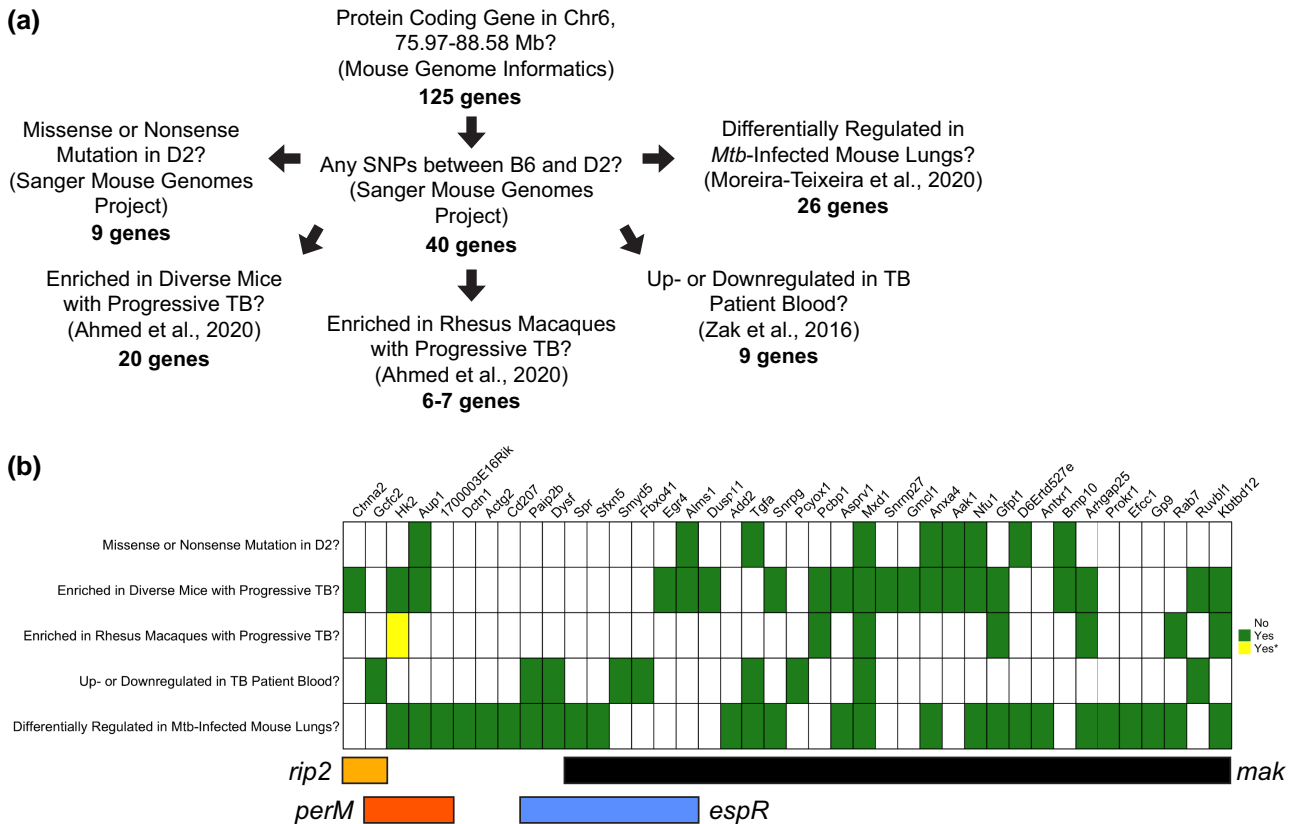


Fig. 7. A bioinformatic pipeline highlights putative candidate genes underlying the QTL on chromosome 6. a) A graphic summarizing the bioinformatic pipeline used to prioritize host gene candidates within each QTL. b) A heatmap representative of the per-gene outcome of 5 distinct criteria: (1) whether or not D2 possesses a missense or nonsense mutation in comparison to B6 according to the Wellcome Sanger Institute Mouse Genomes Project (Sanger MGP) (Adams et al. 2015), (2) whether the gene is significantly enriched in genetically diverse mice progressively infected with *Mtb* (Ahmed et al. 2020), (3) whether the gene is significantly enriched in Rhesus Macaques progressively infected with *Mtb* (Ahmed et al. 2020), (4) whether the gene is significantly upregulated or downregulated in TB patient blood (Zak et al. 2016), and (5) whether the gene is differentially expressed in mouse lungs across variable host genotypes, *Mtb* genotypes, and doses (Moreira-Teixeira et al. 2020). If the answer is no, the space remains blank. Green indicates yes, and yellow indicates yes but cautions that the Rhesus Macaque gene is not a high confidence homolog. Only protein-coding genes with a D2 SNP (as per Sanger MGP) that met at least 1 of the criteria are included in this visual.

protein 1 (*Mxd1*), which is known to be upregulated in murine bone marrow-derived macrophages after infection with the hypervirulent *Mtb* HN878 strain (Roy et al. 2018). Further, we found

a putative host candidate within the QTL mapped by *perM* mutants: ancient ubiquitous protein 1 (*Aup1*). The QTL mapped by the *mak* Tn mutant was enriched for protein-coding genes with

D2-specific SNPs compared with the other QTL within the hotspot. However, the causal locus underlying these QTL may not be protein coding at all. In fact, historical studies of cardiomyopathy in B6 and D2 backcrosses have identified a QTL that was later discovered to be caused by an intronic variant in the tropin-interacting kinase gene *Tnni3k* (Wheeler et al. 2005; Wheeler et al. 2009), which also conferred susceptibility to viral-induced myocarditis (Wiltshire et al. 2011). There are many examples in literature of noncoding causal variants (Hamilton and Yu 2012), yet there remain compelling candidate genes within the chromosome 6 hotspot that could drive the conditional essentiality of these *Mtb* genes in vivo. Altogether, we have leveraged complementary screen data to identify plausible host gene candidates underlying these *host-pathogen* QTL (*hpQTL*).

Discussion

While it is thought that nearly a quarter of the global population is infected with *Mtb* (Houben and Dodd 2016), only 5–10% of *Mtb* infections ultimately result in active TB (Gopal et al. 2014). This paradox has motivated decades of study on human genetic variants that could predict such a lethal prognosis in affected individuals. While Genome-wide association studies (GWAS) among infected populations has identified numerous host loci associated with increased susceptibility to disease, a limited ability to dissect biological mechanism leaves many questions unanswered (Saul et al. 2019). QTL mapping, a parallel systems genetics technique, has been utilized for decades to identify host loci in genetically tractable populations linked to TB susceptibility; *Sst1*, one of the most well-known susceptibility loci, was first discovered through a QTL mapping study in mice (Kramnik et al. 2000). Despite the widespread use of disease traits, including mean survival time, body weight, and bacterial burden, to identify host determinants of disease progression (Lavebratt et al. 1999; Kramnik et al. 2000; Mitsos et al. 2000; Mitsos et al. 2003; Yan et al. 2006), these phenotypes can fail to dissect immunological features that drive distinct *Mtb* pathogenesis in diverse hosts. Here, we describe a screen in which we leveraged the reproducibility, host variation, and phenotypic divergence of the BXD family and the molecular precision of the TnSeq library to identify novel axes of *host-pathogen* interaction during *Mtb* infection. We demonstrate the late (12-week) phenodivergence of the parental B6 and D2 mice; however, we found that CFU serves as a weak predictor of host outcome at the onset of adaptive immunity 4-week postinfection. By utilizing TnSeq as a refined infection trait, we identified bacterial mutants that could distinguish the resistant B6 and susceptible D2 strains at the early 4-week infection timepoint. Finally, we leveraged the differentially required mutants as traits to conduct QTL mapping and identified 140 genome-wide *hpQTL* and a QTL hotspot encompassing a cluster of *Mtb* virulence and cell wall genes. Using this BXD TnSeq platform and a novel candidate prioritization pipeline, we identified viable host gene candidates for future study.

This screen highlights the importance of intentionality in design for deep phenotyping platforms to optimally identify the causal factors underlying gene associations. In contrast to complex macrophenotypes, which require hundreds of mice to sufficiently power causal locus identification for highly polygenic traits, molecular endophenotypes, such as *Mtb* Tn mutant fitness, allow us to learn more about the intricate details of *Mtb* invasion in the early stages of infection, which is particularly helpful in an experimental framework with fewer host genotypes. The strength of TnSeq as a deep

phenotyping platform lies in the redundancy of each *Mtb* gene knockout; within the TnSeq library, each bacterial gene that is non-essential in vitro was perturbed at 4 or more unique Tn insertion sites, resulting in multiple subpopulations of unique replicates of each *Mtb* gene knockout. These mutant replicates taken together paint a robust picture of individual *Mtb* gene requirement in vivo, and multiple host replicates strengthen the reproducibility of each *Mtb* mutant fitness profile. In contrast with our previous TnSeq study that utilized nearly 70 host genotypes (Smith et al. 2022), this TnSeq study includes 21 unique genotypes and 73 mice, including the parental strains B6 and D2. The greater number of Tn mutant *hpQTL* identified in this screen demonstrates the power of a 2-state model to segregate allele effects, as opposed to the 8-state model present in the CC recombinant inbred panel. We, however, cannot eliminate the possibility that the smaller number of host genotypes included in this study did not sufficiently limit statistical noise during QTL mapping analyses.

Using bacterial mutant profiles as endophenotypes, we identified 140 genome-wide significant ($P \leq 0.05$) associations between *Mtb* Tn mutant survival and host genetic loci in a diverse cohort, which may contribute to divergence in clinical outcomes between *Mtb*-resistant and susceptible hosts. These *hpQTL* and the Tn mutant fitness profiles across the BXD panel have been shared with GeneNetwork.org (Chesler et al. 2004; Parker et al. 2017; Ashbrook et al. 2021), an online compendium of BXD phenotypes. Increasing the precision of bacterial traits creates a rich environment for screen-based hypothesis generation in a smaller host population, thereby lowering the cost through lower animal count and less total infection time. This screen serves as a proof of concept that large host populations are not an absolute necessity to uncover novel and biologically meaningful axes of the *host-pathogen* interface. Molecular endophenotyping enables a much more accessible experimental platform to assist in our ongoing struggle against *Mtb*.

From this discovery screen, we have identified several plausible host gene candidates. Variants in *Aup1* represent a possible axis for interaction between *Mtb* and the mammalian host. The acetyltransferase activity of *Aup1* has been shown to be exploited by flaviviruses to trigger autophagy of lipid droplets within the cell, which produces ATP for the virus, thereby promoting viral replication (Zhang et al. 2018). Although not much is known about whether *Mtb* interacts directly with this protein, *Mtb* is one of the few bacteria capable of generating lipid droplets (Daniel et al. 2011). Host lipid droplets, specifically those derived from foamy macrophages, have been shown to promote the production of defensive cytokines during *Mtb* infection (Jaisinghani et al. 2018), implicating a potentially intriguing locus for *host-pathogen* interaction. The other high priority candidate *Mxd1* encodes the protein MAD, which competes with MYC to bind with MAX, forming a transcriptional repressor. This mechanism has led *Mxd1* to be considered a putative tumor suppressor gene. *Mxd1* has been shown to be upregulated in murine bone marrow-derived macrophages upon initial infection with the hypervirulent *Mtb* HN878 strain (Roy et al. 2018) and to regulate the fitness of murine dendritic cells (Anderson et al. 2020), yet the function of *Mxd1* in response to *Mtb* has yet to be determined. Together, these candidates provide feasible next steps for mechanistic interrogation of these loci.

While the functions of many mycobacterial genes remain unknown (Whitaker et al. 2020), this Tn mutant screen within the BXD family builds directly upon a previous screen in the CC panel (Smith et al. 2022) to further articulate the conditional necessity of many canonically “nonessential” *Mtb* genes within a spectrum of

host microenvironments. A substantial number of *Mtb* genes known to be nonessential in B6 mice prove to be essential in a subset of these genetically diverse hosts, suggesting that these bacterial genes play an active yet undiscovered role in pathogenesis under specific immunological conditions. These new host conditions have been shared with MtbTnDB (Jinich et al. 2021), a central repository encompassing over 60 published *Mtb* TnSeq screens for ease of browsing and interscreen comparison. For mycobacterial researchers exploring *Mtb* genes traditionally thought to be nonessential in vivo, this work challenges such a notion and offers a new model that will provide insight into the host–pathogen dynamic underlying this conditional in vivo essentiality.

Data availability

All relevant data to support the findings of this study are located within the paper and supplementary files. Genome sequence data is available in the NCBI Gene Expression Omnibus (GEO), accession number GSE234093. All QTL mapping files are publicly available at: https://github.com/SmithLabDuke/Meade_et_al_BXD_TnSeq. Tn mutant fitness profiles within each BXD genotype and parental genotypes are publicly available on MtbTnDB (<https://www.mtbtndb.app/>). BXD phenotypes are available on GeneNetwork (<https://www.genenetwork.org>).

Supplemental material available at G3 online.

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Conflicts of interest

The author(s) declare no conflicts of interest.

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