Genome-Wide Essentiality Analysis of *Mycobacterium abscessus* by Saturated Transposon Mutagenesis and Deep Sequencing

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ABSTRACT *Mycobacterium abscessus* is an emerging opportunistic human pathogen that naturally resists most major classes of antibiotics, making infections difficult to treat. Thus far, little is known about *M. abscessus* physiology, pathogenesis, and drug resistance. Genome-wide analyses have comprehensively catalogued genes with essential functions in *Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. *hominissuis* (here, *M. avium*) but not in *M. abscessus*. By optimizing transduction conditions, we achieved full saturation of TA insertion sites with Himar1 transposon mutagenesis in the *M. abscessus* ATCC 19977[T] genome, as confirmed by deep sequencing prior to essentiality analyses of annotated genes and other genomic features. The overall densities of inserted TA sites (85.7%), unoccupied TA sites (14.3%), and nonpermissive TA sites (8.1%) were similar to results in *M. tuberculosis* and *M. avium*. Of the 4,920 annotated genes, 326 were identified as essential, 269 (83%) of which have mutual homology with essential *M. tuberculosis* genes, while 39 (12%) are homologous to genes that are not essential in *M. tuberculosis* and *M. avium*, and 11 (3.4%) only have homologs in *M. avium*. Interestingly, 7 (2.1%) essential *M. abscessus* genes have no homologs in either *M. tuberculosis* or *M. avium*, two of which were found in phage-like elements. Most essential genes are involved in DNA replication, RNA transcription and translation, and posttranslational events to synthesize important macromolecules. Some essential genes may be involved in *M. abscessus* pathogenesis and antibiotics response, including certain essential tRNAs and new short open reading frames. Our findings will help to pave the way for better understanding of *M. abscessus* and benefit development of novel bactericidal drugs against *M. abscessus*.

IMPORTANCE Limited knowledge regarding *Mycobacterium abscessus* pathogenesis and intrinsic resistance to most classes of antibiotics is a major obstacle to developing more effective strategies to prevent and mitigate disease. Using optimized procedures for Himar1 transposon mutagenesis and deep sequencing, we performed a comprehensive analysis to identify *M. abscessus* genetic elements essential for *in vitro* growth and compare them to similar data sets for *M. tuberculosis* and *M. avium* subsp. *hominissuis*. Most essential *M. abscessus* genes have mutual homology with essential *M. tuberculosis* genes, providing a foundation for leveraging available knowledge from *M. tuberculosis* to develop more effective drugs and other interventions against *M. abscessus*. A small number of essential genes unique to *M. abscessus* deserve further attention to gain insights into what makes *M. abscessus* different from other mycobacteria. The essential genes and other genomic features such as short open reading frames and noncoding RNA identified here will provide useful information for future study of *M. abscessus* pathogenicity and new drug development.

Mycobacterium abscessus complex comprises the largest group of rapidly growing nontuberculosis mycobacteria (1). It causes chronic lung infection in individuals with cystic fibrosis (CF) and other structural lung diseases, as well as skin and skin structure infections (1–3). Treatment of M. abscessus lung infection is difficult. Cure rates are approximately 30% despite long courses of treatment with poorly tolerated regimens (4). The poor efficacy of existing treatments is attributable in part to the high degree of intrinsic resistance to most major classes of antibiotics, including most anti-tuberculosis drugs (2, 5, 6). Safer, more effective drugs are urgently needed. Genome-wide essentiality analyses could help to identify new drug targets for development of novel drugs targeting M. abscessus.

M. abscessus is ubiquitous in the environment, including soil and water (4). Little knowledge exists regarding genome-wide elements that are essential for M. abscessus viability in the inanimate environment or its ability to cause disease. These knowledge gaps result in part from the limited availability and/or use of genetic tools (7–9). Comprehensive predictions of essential genes and other genetic elements, such as short open reading frames (ORFs), noncoding RNA, and tRNA in Mycobacterium tuberculosis and Mycobacterium avium subsp. hominissuis (here, M. avium), made using saturated transposon (Tn) mutant pools and deep sequencing were recently reported (10, 11). Transposon sequencing (Tn-Seq) is a powerful tool to determine the essentiality of genes or other genomic features for growth and survival under experimental conditions (10–13). Essential and conditionally essential genes may represent ideal targets for novel drugs or important virulence factors to target with interventions in order to better treat or prevent M. abscessus infections.

Here, we optimized conditions for Himar1 Tn mutagenesis of M. abscessus subsp. abscessus strain ATCC 19977^T to generate saturated Tn mutant pools and then prepared fully saturated DNA libraries for deep sequencing. Comprehensive genomic analysis was performed using a Hidden Markov Model (HMM) to predict essentiality of annotated genes and other genomic features for in vitro growth. We also compared essential genes of M. abscessus to those of M. tuberculosis and M. avium to characterize commonalities and differences in essentiality between those pathogens. Our findings provide insights for understanding M. abscessus pathogenesis and pave the way for developing safer, more effective drugs to treat M. abscessus infections.

RESULTS

Optimization of Tn mutagenesis. To generate fully saturated Himar1 Tn mutant pools, we optimized previously described protocols step by step to test the impact of variables, including the multiplicity of infection (MOI) and transduction time and speed, as well as the bacterial density (10, 11). Transduction of M. abscessus at an MOI of 20:1 and incubation for 4 h in a 37°C shaker at 180 rpm yielded the highest Tn insertion frequency (Fig. 1A to C). A cell density of 8.2×10^{10} CFU/ml obtained by concentrating a culture at early stationary phase yielded a higher transformation frequency (9.3×10^{-6}) than a density of 1.5×10^9 CFU/ml under these conditions (Fig. 1D). A total of 10 independent Tn mutant pools were created, each containing 3.4×10^5 to 1×10^6 insertion events. When all 10 Tn mutant pools were combined, 7,454,000 independent insertion events were identified. Spontaneous kanamycin-resistant mutants occurred at a frequency of 1 to 3% among the Tn mutant pools, as observed in M. avium (11). To examine the quality of the pools, randomly selected colonies were subjected to PCR and enzyme digestion to detect the Tn and the Tn-genome junction region using primers listed in Table S1 in the supplemental material. All 40 colonies selected contained the 850-bp DNA fragment of the kanamycin resistance gene of the Himar1 Tn. Ten colonies were further analyzed and confirmed to harbor a single DNA fragment containing the junction of the Tn and a genetic element of M. abscessus ATCC 19977^T, indicating a single Tn insertion event (data not shown).

Saturation and essentiality analysis of TA sites. To achieve full coverage of TA sites on the genome, we performed deep sequencing of Tn DNA libraries prepared in triplicate from each of the 10 independent Tn mutant pools using unique sequencing
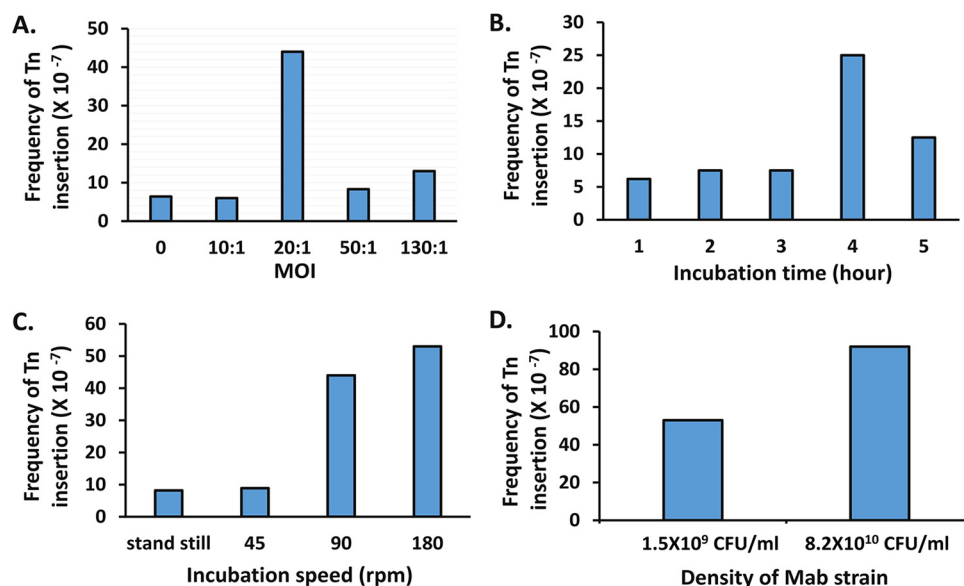
FIG 1 Optimization of experimental conditions for generation of a saturated Tn mutant pool using the Himar1 Tn. (A) A culture containing 1.5×10^9 CFU/ml of *M. abscessus* ATCC 19977^T was infected at different MOIs (ratio of ΦmycomarT7 phage PFU to *M. abscessus* CFU counts) and incubated at 37°C in a shaker at 90 rpm for 3 h. (B) A culture containing 1.5×10^9 CFU/ml of *M. abscessus* was infected with the same phage at an MOI of 10:1 and incubated at 37°C in a shaker at 90 rpm for different periods of time. (C) A culture containing 1.5×10^9 CFU/ml of *M. abscessus* was infected with the same phage at an MOI of 20:1 and incubated at 37°C in a shaker at different speeds for 4 h. (D) Cultures containing different concentrations of *M. abscessus* were infected with the same phage at an MOI of 20:1 and incubated at 37°C in a shaker at 180 rpm for 4 h. All experiments were repeated once and showed similar results.

primers listed in Table S1. The resulting 30 Tn DNA libraries yielded an average of 4.5 million unique Tn-genome junctions (termed "template counts"). The average template count for each TA site is shown in Data Set S1A. Results of statistical analyses of the Tn DNA libraries after deep sequencing are summarized in Table S2. The *M. abscessus* ATCC 19977^T genome consists of a 5,067,172-bp circular chromosome (14) containing 91,240 TA sites. The average density of Tn insertions into TA sites for each individual Tn DNA library was 65%, but the cumulative density for each Tn pool increased to 74 to 78% after combining results from triplicate DNA libraries (see Table S2). Each pool contained 67,518 to 71,167 unique mutants. The overall insertion density achieved after cumulating the inserted TA sites identified in any of the 30 Tn DNA libraries was 85.7% (78,165 of 91,240 TA sites) (Fig. 2A). The cumulative insertion density reached a plateau after combining ≥5 of the 10 Tn mutant pools, indicating full saturation of TA sites available for insertion. Most TA sites (63.8% [58,181/91,240]) were detected in all 10 Tn pools with a mean read count of 229 per TA site, while only small proportions of inserted TA sites (i.e., 1.7 to 4.9%) with low mean read counts (i.e., 1.4 to 21) were observed in only 1 to 9 Tn pools (Fig. 2B and C). Tn insertions were not detected in the remaining 14.3% (13,075/91,240) of TA sites in any of the 10 pools (Fig. 2B), similar to results observed in *M. tuberculosis* (15.7%) and *M. avium* (16.5%) (10, 11).

The essentiality of each TA site was defined using an HMM (15, 16), a statistical model that considers read counts both at a given TA site and distributed over surrounding sites and dynamically adjusts probability distributions over states using geometric distributions to obtain locally consistent interpretations of essentiality across the genome. As such, TA sites with no insertions in nonessential regions are tolerated because neighboring sites have insertions. In contrast, if a consecutive sequence of TA sites with no insertions is long enough, the most probable state of that sequence will be assigned as essential (16). Read counts were modeled as having geometric distributions conditioned on four different states of essentiality for TA sites: essential (ES),
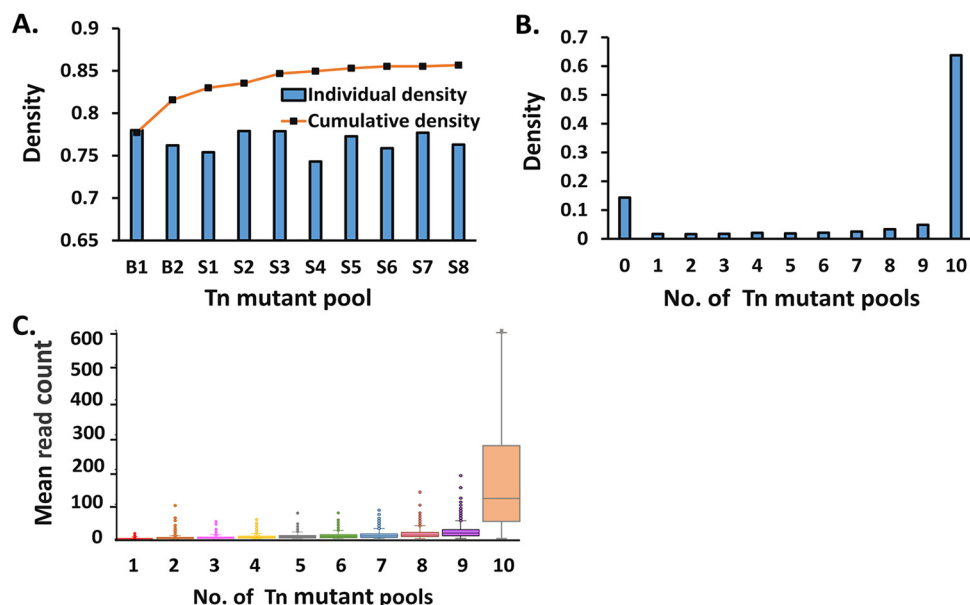
FIG 2 Evaluation of saturated TA sites in Tn mutant pools by deep sequencing. (A) Cumulative density of TA sites with insertions (orange line) obtained by combining independent Tn mutant pools (blue bars represent density of each individual pool). (B) Density of TA sites with insertions represented as the number of Tn mutant pools in which each particular insertion event was detected. (C) Mean read counts for TA sites with insertions detected by deep sequencing according to the number of Tn mutant pools containing that particular insertion.

nonessential (NE), and causing either a growth defect (GD) or growth advantage (GA) upon Tn insertion. Parameters for expected read count distributions for each state were set relative to the mean value of nonempty read counts (16). In the *M. abscessus* genome, 6.8 and 79.6% of TA sites were defined as ES and NE, respectively, for *in vitro* growth; 10.9 and 2.7% were defined as GA and GD, respectively, when disrupted (see Data Set S1A). The inferred essentiality of TA sites was used to determine the essentiality of individual coding sequences and non-ORF genomic features. The same analysis was performed on the plasmid pMAB23 harbored by *M. abscessus* ATCC 19977[T].

To better understand why some TA sites had few or no insertions or lower read counts in some number of pools (Fig. 2B and C), the TA site motif (G/C)GNTANC(G/C) identified in prior *M. tuberculosis* and *M. avium* studies as being less permissive to Himar1 insertion (10, 11) was investigated in the *M. abscessus* genome. It was greatly enriched in a set of 6,000 putative nonpermissive TA sites lacking Tn insertions compared to a set of putative permissive sites with the highest 25% of read counts. This nonpermissive motif was identified at 8.1% (7,425/91,240) of TA sites (see Data Set S1A), similar to the frequency in *M. tuberculosis* (9%) (10). Of TA sites with the nonpermissive motif, 60.9% had no Tn insertion detected in any mutant pool. Furthermore, the probability of the nonpermissive motif appearing at a TA site was inversely proportional to the number of pools in which Tn insertions were detected at that TA site (see Table S3), confirming that the nonpermissive motif was associated with lower Tn insertion frequency and lower read counts in the *M. abscessus* genome, as in *M. tuberculosis* and *M. avium* (10, 11). Remarkably, 23% of the 13,075 TA sites without a Tn insertion detected could not be explained by either a prediction of essentiality in the HMM or presence of the nonpermissive motif (Fig. 3).

Essentiality analysis of annotated genes. Of 4920 annotated genes, 326 were identified as ES, 144 as GD, 589 as GA, and 3855 as NE (Table 1). Detailed information regarding essentiality of each gene is shown in Data Set S1B. Only six genes lacked TA loci and therefore could not be assessed by our approach. A homology comparison between proteins encoded by annotated genes of *M. abscessus* ATCC 19977[T]
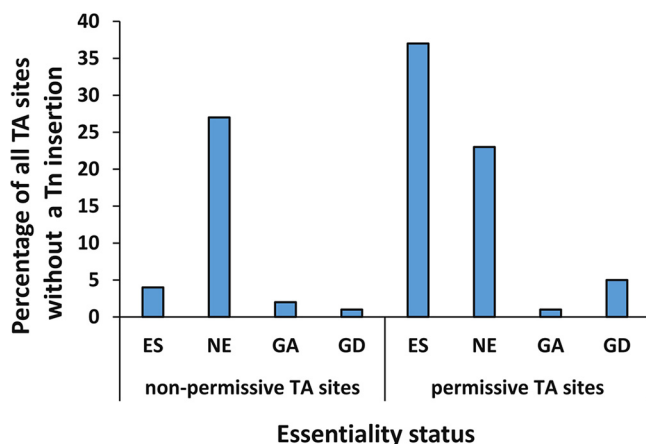
FIG 3 Distribution of 13,075 TA sites devoid of Tn insertions in any of 10 saturated Tn mutant pools according to the presence (4,525 TA sites) or absence (8,550 TA sites) of a previously described nonpermissive motif and the predicted essentiality status. TA sites were predicted by the four-state HMM (ES, essential; GD, growth defect when mutated; GA, growth advantage when mutated; NE, nonessential).

($n = 4,920$), *M. tuberculosis* H37Rv ($n = 4,018$), and *M. avium* strain MAC109 ($n = 4,704$) revealed that 44.1% (2,170/4,920) of *M. abscessus* genes shared common orthologs with both *M. tuberculosis* and *M. avium* genes, and small numbers of genes were only homologous to *M. tuberculosis* (2.5%, 122/4,920) or *M. avium* (9.7%, 477/4,920); 43.6% (2,145/4,920) of *M. abscessus* genes had no significant homology to *M. tuberculosis* or *M. avium* genes.

Most essential *M. abscessus* genes are orthologs of *M. tuberculosis* genes required for *in vitro* growth. A homology comparison of 326 *M. abscessus* essential genes with 461 and 270 essential genes from *M. tuberculosis* and *M. avium*, respectively (10, 11), is shown in Fig. 4. A total of 41.4% (135/326) of *M. abscessus* essential genes share mutual homology with *M. tuberculosis* and *M. avium* (see Table S4), and 41.1% have essential orthologs only in *M. tuberculosis* (see Table S5), while 3.4% (11/326) have essential orthologs only in *M. avium* (see Table S5). Interestingly, 12% (39/326) of *M. abscessus* essential genes are homologous to genes that are not essential in *M. tuberculosis* (37 genes) or *M. avium* (2 genes) (Table 2). For example, *MAB_3090c* encoding dihydrofolate reductase was defined as essential in *M. abscessus*, but its *M. tuberculosis* ortholog *Rv2763c* is not essential (10). Moreover, 2.1% (7/326) of *M. abscessus* essential genes have no homology with *M. tuberculosis* or *M. avium* genes (Table 3).

A large number of essential genes are involved in DNA replication, RNA transcription and translation, protein folding, cell wall organization and regulation of cell shape. For example, *MAB_3869c* encodes the ortholog of the DNA-directed RNA polymerase beta chain RpoB in *M. tuberculosis* (see Table S4), the target of first-line antituberculosis (anti-TB) rifamycin drugs (17). However, this class has limited utility for treatment of *M. abscessus* infections due to intrinsic resistance (18, 19). Another large group of essential genes is associated with biosynthesis and transport of nucleotides, amino acids, fatty acids and cell wall components. All 19 genes encoding tRNA synthetases for transfer of 20 common amino acids are essential (Table 2 and Table S4). *M. abscessus* genes responsible for energy support, including *MAB_1448-MAB_1453* encoding the ATP synthase operon are also essential, including *atpE* (*MAB_1448*), the target of bedaquiline (see Tables S4 and S5), which potently inhibits ATP generation in *M. tuberculosis* (20) and in *M. avium* and *M. abscessus* (21–24).

Essentiality analysis of genes involved in pathogenesis. Functional analysis and homology comparisons identified 49 *M. abscessus* genes potentially involved in *M. abscessus* virulence, some of which were referenced from a previous study by Ripoll et al. (14) (Table 4). Of these, only four are essential for *in vitro* growth. *MAB_1933c* encodes glutamine synthetase, type I (GlnA1) (Table 4), which catalyzes ATP-dependent

TABLE 1 Summary of essentiality analysis of *M. abscessus* ATCC 19977^T genome by Tn-Seq[a]

Genomic feature	Total no.	No. of genomic feature by assigned essentiality status				
		ES	GD	GA	NE	NA (without TA site)
ORF	4,920	326	144	589	3,855	6
sORF	126	5	0	15	89	17
ncRNA	36	4	2	5	19	6
tRNA	47	10	0	4	31	2
rRNA	3	3	0	0	0	0
Rho-independent terminator	750	20	5	46	359	320
5′ UTR	1,503	35	26	194	991	257
Promoter region	3,374	83	46	557	2,671	17

[a]ES, essential; GD, growth defect when mutated; GA, growth advantage when mutated; NE, nonessential; NA, not assessable using our approach due to genomic features without any TA site.

assimilation of ammonia into glutamate to form glutamine in *M. tuberculosis* (25). This process accounts for 15% of total ATP consumption in *Escherichia coli* (26, 27). *MAB_1077* encodes the two-component sensor kinase MprB (Table 4). In *M. tuberculosis*, MprAB, encoded by *Rv0981* and *Rv0982*, interacts with chaperone protein DnaK (Hsp70) to properly maintain protein homeostasis in the extracytoplasmic compartment in response to cell envelope stress (28). The other two essential genes, *MAB_0118c* and *MAB_3473c* (Table 4), encode a probable Mn-dependent superoxide dismutase and an SsrA-binding protein, respectively. The former secreted protein protects mycobacteria from reactive oxygen species, especially in macrophages (29–31). The latter is responsible for recycling stalled ribosomes and tagging incomplete nascent proteins for degradation during *trans*-translation (32, 33). *M. abscessus* only contains two type VII secretion (T7S) systems, including ESX-3 and ESX-4 loci, whereas *M. tuberculosis* harbors five T7S systems (14, 34). The essentiality and the homology comparisons for the ESX-3 and ESX-4 genes are shown in Table S6. The *M. abscessus* genes in these loci were defined as nonessential under our test conditions while, in *M. tuberculosis*, genes encoding ESX-3 were essential and those encoding ESX-4 were nonessential. The *M. abscessus* genes were homologous to *M. tuberculosis* genes except that nonessential *MAB_3760* encoding EccE4 is not found in most other interrogated mycobacteria, with the exception of *M. chelonae* and *Mycobacterium immunogenum*, thereby potentially distinguishing the *M. abscessus* ESX-4 system from that of most other mycobacteria (35, 36).

Essentiality analysis of genes involved in responses to antibiotics. Table 5 lists 58 *M. abscessus* genes possibly associated with antibiotic responses (including drug targets, drug-modifying enzymes, drug transporters, and TetR family regulators), identified through the result of comparative genomic and functional analysis. Only 16
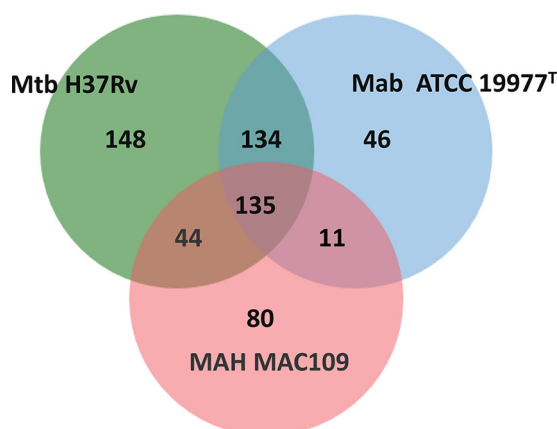
FIG 4 Homology comparison of essential genes among *M. abscessus* (Mab) ATCC 19977^T, *M. tuberculosis* (Mtb) H37Rv, and *M. avium* subsp. *hominissuis* (MAH) MAC109.

TABLE 2 Essential *M. abscessus* genes homologous to nonessential genes in *M. tuberculosis* H37Rv or *M. avium* MAC109

Gene[a]	Description
MAB_0037c	Possible serine/threonine phosphatase Ppp
MAB_0343	Aspartate kinase
MAB_0344	Aspartate-semialdehyde dehydrogenase Asd
MAB_0487	Probable cold shock protein A CspA
MAB_1513	Putative holo-[acyl-carrier-protein] synthase
MAB_1607	Possible ribonuclease E Rne
MAB_1672	GTP-binding protein Era homolog
MAB_2005	Putative cell division protein FtsW
MAB_2096c	Putative MutT/NUDIX-like protein (homologous to *M. avium* gene)
MAB_2116	Cysteinyl-tRNA synthetase CysS
MAB_2159	Conserved hypothetical protein (RNA methyltransferase?)
MAB_2778c	Probable phosphoglycerate kinase PGK
MAB_2779c	Glyceraldehyde-3-phosphate dehydrogenase, type I
MAB_2848c	Probable shikimate-5-dehydrogenase AroE
MAB_2879c	Probable protein-export membrane protein SecF
MAB_3090c	Dihydrofolate reductase DfrA
MAB_3110	Probable iron dependent transcriptional repressor FeoA
MAB_3167c	Putative penicillin-binding protein
MAB_3259c	Phosphopantetheine adenylyltransferase CoaD
MAB_3342c	Glutamyl-tRNA(Gln) amidotransferase subunit C GatC
MAB_3772c	30S ribosomal protein S11
MAB_3793c	50S ribosomal protein L15
MAB_3794c	50S ribosomal protein L30
MAB_3804c	30S ribosomal protein S14P/S29E
MAB_3812c	50S ribosomal protein L29
MAB_3991c	Possible uroporphyrin-III C-methyltransferase
MAB_4145	Probable α,α-trehalose-phosphate synthase
MAB_4953c	Membrane protein OxaA
MAB_4954c	Ribonuclease P protein component
MAB_4955c	50S ribosomal protein L34

[a]Genes for hypothetical proteins: MAB_1062, MAB_1669, MAB_2403, MAB_2404, MAB_2751, MAB_2893c, MAB_4077, MAB_4318 (homologous to a *M. avium* gene), and MAB_4471.

genes were classified as essential, virtually all of them being drug targets in *M. tuberculosis* that have been validated *in vivo*. Among them, *MAB_0019* and *MAB_0006* encode DNA gyrase subunits A and B, respectively (Table 5), comprising the sole DNA topoisomerase responsible for DNA decatenation, relaxation, and supercoiling. GyrA is the target of fluoroquinolones such as moxifloxacin in TB treatment (37). The *in vivo* efficacy of moxifloxacin against *M. abscessus* remains uncertain despite it showing activity *in vitro* (38, 39). The clinical candidate SPR720 targets GyrB and is being developed for both *M. tuberculosis* and *M. abscessus* infections (40–42). *MAB_2722c* encodes the NADH-dependent enoyl-(acyl-carrier-protein) reductase that catalyzes the last step in the fatty acid elongation cycle for mycolic acid biosynthesis and *MAB_0185c* encodes arabinosyltransferase B that is necessary for arabinogalactan biosynthesis (Table 5). These enzymes are targets of the first-line anti-TB drugs isoniazid and ethambutol, respectively (43, 44), which are not recommended for treating *M. abscessus* infections due to intrinsic resistance (45). Among all MmpL family members, only *MAB_4508* encoding the trehalose monomycolate flippase MmpL3 is essential in *M. abscessus* (46)

TABLE 3 Essential *M. abscessus* genes with no homology to *M. tuberculosis* H37Rv or *M. avium* MAC109

Gene[a]	Description	Phage-like element coordinates
MAB_0222c	Putative DNA-binding protein	4909957–4959626
MAB_3419	NH_3-dependent NAD^+ synthetase NadE	
MAB_4828c	Hypothetical protein	233621–247981

[a]Genes for hypothetical proteins: MAB_0210, MAB_1556, MAB_2350c, and MAB_3624c.

TABLE 4 Essentiality analysis and homology comparison of *M. abscessus* genes possibly involved in pathogenesis[a]

Homology search	Gene	Description	Call
Homology with *M. tuberculosis*	MAB_0942	Putative transcriptional regulator, AraC family	NE
	MAB_0945	Putative drug resistance transporter, EmrB/QacA family	NE
	MAB_3997c	Probable transcriptional regulatory protein	NE
Homology with *M. tuberculosis* and *M. avium*	MAB_0046	Probable PE family protein	NE
	MAB_0115c	Hypothetical protein	GA
	MAB_0118c	Probable superoxide dismutase [Mn]	ES
	MAB_0169c	Putative exported repetitive protein precursor	GA
	MAB_0176	Antigen 85A precursor	NE
	MAB_0283c	Hypothetical protein	NE
	MAB_0474	Hypothetical protein	NE
	MAB_0545	Protein lsr2 precursor	NE
	MAB_0580	Probable acyl-CoA dehydrogenase FadE	NE
	MAB_0593c	Probable acyl-CoA dehydrogenase FadE	NE
	MAB_0594c	Probable acyl-CoA dehydrogenase FadE	NE
	MAB_0613	Putative cytochrome P450	NE
	MAB_0615	Putative acyl-CoA dehydrogenase	NE
	MAB_0673	Putative DNA-binding response regulator PhoP	NE
	MAB_0869c	Probable resuscitation-promoting factor RpfA	NE
	MAB_0885c	Hypothetical lipoprotein LpqH precursor	NE
	MAB_0956c	Probable transcriptional regulatory protein PrrA	NE
	MAB_1069c	Probable enoyl-CoA hydratase/isomerase	NE
	MAB_1077	Probable two-component sensor kinase MprB	ES
	MAB_1918	Conserved hypothetical protein (possible hydrolase)	GA
	MAB_1933c	Probable glutamine synthetase, type I GlnA1	ES
	MAB_1945c	2-Oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	GD
	MAB_2124	Putative phenyloxazoline synthase MbtB	NE
	MAB_2140	NADH-quinone oxidoreductase, G subunit NuoG	NE
	MAB_2231c	Hypothetical PE family protein	NE
	MAB_2262c	Hypothetical ABC transporter ATP-binding protein	NE
	MAB_2379	Hypothetical lipoprotein LpqH precursor	NE
	MAB_2470c	Catalase/peroxidase KatG	GA
	MAB_2728c	Hypothetical invasion protein Inv1	NE
	MAB_3029	Iron-dependent repressor IdeR	GA
	MAB_3428c	Probable RNA polymerase sigma-C factor	NE
	MAB_3473c	SsrA-binding protein	ES
	MAB_3726	Putative transcriptional regulator, WhiB family	GA
	MAB_3891c	Probable transcriptional regulator, LuxR family	NE
	MAB_4083c	Heparin-binding hemagglutinin (adhesin)	GA
	MAB_4095c	Isocitrate lyase AceA	NE
	MAB_4149c	Hypothetical MCE-family protein LprN	NE
	MAB_4158	Probable acyl-CoA dehydrogenase FadE	NE
	MAB_4159	Probable acyl-CoA dehydrogenase	NE
	MAB_4184c	Superoxide dismutase [Cu-Zn] precursor	NE
	MAB_4251	Conserved hypothetical protein (peptidase?)	NE
	MAB_4336	Probable acyl-CoA dehydrogenase FadE	NE
	MAB_4402	Heat shock protein Hsp20	NE
	MAB_4407c	Putative alkylhydroperoxidase AhpD	NE
	MAB_4408c	Putative alkylhydroperoxidase AhpC	NE
Homology with *M. avium*	MAB_3123	Putative acyl-CoA dehydrogenase	NE

[a]CoA, coenzyme A; ES, essential; GD, growth defect when mutated; GA, growth advantage when mutated; NE, nonessential.

(Table 5). The β-lactamase Bla$_{Mab}$ encoded by *MAB_2875* is a major cause of the limited efficacy of β-lactams against *M. abscessus* (47), although a homology search identified 18 nonessential genes encoding possible β-lactamases worthy of investigation, especially those having no homology with either *M. tuberculosis* or *M. avium* (see Table S7).

Essentiality analysis of genes involved in peptidoglycan metabolism. Mycolyl-arabinogalactan-peptidoglycan complex is the key structure for the mycobacterial cell wall that contributes to the intrinsic resistance to a number of antibiotics. Consequently, it is a major target for drug development (48). Table 6 lists genes

TABLE 5 Essentiality analysis and homology comparison of *M. abscessus* genes possibly involved in responses to existing antibiotics[a]

Homology	Gene	Description	Call
No homology	MAB_0856c	Putative transcriptional regulator, TetR family	NE
	MAB_0591	Probable rifampin ADP-ribosyl transferase	NE
	MAB_1430c	Putative transcriptional regulator, TetR family	NE
	MAB_1496c	Putative FAD-binding monooxygenase	NE
	MAB_1497c	Putative regulatory protein, TetR family	NE
	MAB_2380c	Putative transcriptional regulator, TetR family	NE
	MAB_2385	Probable streptomycin phosphotransferase	NE
	MAB_2685	Putative transcriptional regulator, TetR family	GA
	MAB_2989	Probable chloramphenicol acetyltransferase	NE
	MAB_4320c	Putative transcriptional regulator, TetR family	NE
	MAB_4384	TetR regulator	NE
	MAB_4532c	Gcn5-related *N*-acetyltransferase (GNAT) Eis2	NE
Homology with *M. tuberculosis*	MAB_0163c	Probable phosphotransferase	NE
	MAB_0945	Putative drug resistance transporter, EmrB/QacA family	NE
	MAB_1448	ATP synthase C chain AtpE	ES
	MAB_1858	Probable ABC transporter antibiotic-transport ATP-binding protein	NE
	MAB_1859	Probable ABC transporter antibiotic-transport integral membrane protein	NE
	MAB_1860	Probable ABC transporter antibiotic-transport integral membrane protein	NE
	MAB_2297	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(41)	NE
	MAB_2875	β-Lactamase precursor (Penicillinase)	NE
	MAB_3080c	Dihydrofolate reductase DfrA	ES
	MAB_4283c	Conserved hypothetical protein (isoniazid-inducible gene protein IniA?)	NE
	MAB_4395	Aminoglycoside 2'-N-acetyltransferase	NE
Homology with *M. tuberculosis* and *M. avium*	MAB_0006	DNA gyrase (subunit B) GyrB (DNA topoisomerase)	ES
	MAB_0019	DNA gyrase (subunit A) GyrA (DNA topoisomerase)	ES
	MAB_0173	Prenyltransferase family protein UbiA	ES
	MAB_0180	Polyketide synthase PKS13	ES
	MAB_0185c	Probable arabinosyltransferase B	ES
	MAB_0189c	Probable arabinosyltransferase C	GD
	MAB_0192c	Probable oxidoreductase	ES
	MAB_0408c	Probable bifunctional membrane-associated penicillin-binding protein PonA2/glycosyl transferase	GA
	MAB_1134c	Probable membrane protein MmpL	NE
	MAB_2301	Probable membrane protein MmpL	GA
	MAB_1359c	Putative ABC transporter, ATP-binding protein	NE
	MAB_1409c	Multidrug efflux transporter Tap	NE
	MAB_1472c	Putative nicotinamidase/pyrazinamidase	NE
	MAB_1560	Probable ABC transporter (macrolide-transport) ATP-binding protein	ES
	MAB_1689	Probable daunorubicin resistance ABC transporter ATP-binding subunit DrrA	NE
	MAB_1877c	3-Oxoacyl-[acyl-carrier-protein] synthase 1 KasA	ES
	MAB_2108	Probable undecaprenyl-diphosphatase (Bacitracin resistance protein)	NE
	MAB_2208c	Hypothetical protein	GA
	MAB_2319c	Probable lysyl-tRNA synthetase 2 LysX	NE
	MAB_2415c	Conserved hypothetical protein (penicillinase repressor?)	GA
	MAB_2643c	Tryptophan synthase, alpha subunit TrpA	ES
	MAB_2644c	Tryptophan synthase, beta subunit TrpB	ES
	MAB_2705c	Isoleucyl-tRNA synthetase IleS	ES
	MAB_2722c	Enoyl-(acyl-carrier-protein) reductase (NADH)	ES
	MAB_3508c	Putative transcriptional regulator	NA
	MAB_2299c	TetR regulator	GA
	MAB_4382c	Putative membrane protein MmpL5	NE
	MAB_4383c	Putative membrane protein MmpS5	NE
	MAB_4482	Putative phosphotransferase	NE
	MAB_4659	Conserved hypothetical protein (phosphoribosyltransferase?)	NE
	MAB_4923	Leucyl-tRNA synthetase	ES
Homology with *M. avium*	MAB_1135c	Probable membrane protein MmpS	GA
	MAB_2300	Probable membrane protein MmpS	GA
	MAB_3449c	Putative transporter	NE
	MAB_4508	Putative membrane protein MmpL	ES

[a]ES, essential; GD, growth defect when mutated; GA, growth advantage when mutated; NE, nonessential.

TABLE 6 Essentiality analysis and homology comparison of genes associated with peptidoglycan biosynthesis and remodeling in *M. abscessus* ATCC 19977[T] and *M. tuberculosis* H37Rv[a]

Category	*M. tuberculosis* gene	Call	*M. abscessus* gene	Call	Description of *M. abscessus* gene
Resuscitation promoting factors	*rpfA* (Rv0867c)	NE	MAB_0869c	NE	Probable resuscitation-promoting factor RpfA
	rpfB (Rv1009)	NE	MAB_1130	NE	Hypothetical protein
	rpfC (Rv1884c)	NE	MAB_4080c	NE	Hypothetical protein
	rpfD (Rv2389c)	NE	NA		NA
	rpfE (Rv2450)	NE	MAB_1597	GA	Hypothetical protein
Penicillin-binding proteins and noncanonical transglycosylases	*ponA1* (Rv0050)	GA	MAB_4901c	GA	Penicillin-binding protein
	ponA2 (Rv3682)	NE	MAB_0408c	GA	Probable bifunctional membrane-associated penicillin-binding protein PonA2/glycosyl transferase
	pbpA (Rv0016c)	NE	MAB_0035c	GA	Probable penicillin-binding protein PbpA
	pbpB (Rv2163c)	ESD	MAB_2000	ES	Probable penicillin-binding membrane protein PbpB
	Rv2864c	NE	MAB_3167c	ES	Putative penicillin-binding lipoprotein
	dacB1 (Rv3330)	NE	MAB_3681	NE	Probable penicillin-binding protein DacB1
	dacB2 (Rv2911)	NE	MAB_3234	GA	Probable D-alanyl-D-alanine carboxypeptidase DacB2
	Rv3627c	GD	MAB_0519	NE	Peptidase S13 (D-alanyl-D-alanine carboxypeptidase)
	ftsW (Rv2154)	ES	MAB_2005	ES	Putative cell division protein
	rodA (Rv0017)	NE	MAB_0036c	GA	Probable cell division protein
	MSMEG_1900	NE	MAB_2019	NE	Putative VanY-type carboxypeptidase
Endopeptidases	Rv0024	NE	NA		NA
	ripA (Rv1477)	ESD	MAB_2728	NE	Hypothetical invasion protein Inv1
	ripB (Rv1478)	NE	MAB_2727c	NE	Hypothetical invasion protein Inv2
	ripD (Rv1566c)	NE	MAB_2474	NE	Hypothetical protein
	Rv2190c	NE	MAB_1974	GA	Putative secreted protein
L,D-Transpeptidases	*ldt*$_{Mt1}$ (Rv0116c)	NE	MAB_3165c	GA	Hypothetical protein
	ldt$_{Mt2}$ (Rv2518c)	NE	MAB_1530	GA	Probable conserved lipoprotein LppS
	ldt$_{Mt3}$ (Rv1433)	NE	MAB_4775c	NE	Hypothetical protein
	ldt$_{Mt4}$ (Rv0192)	NE	MAB_4537c	NE	Hypothetical protein
	ldt$_{Mt5}$ (Rv0482)	NE	MAB_4061c	NE	Hypothetical protein
Amidases	*ami1* (Rv3717)	NE	MAB_0318c	GA	Hypothetical protein
	ami2 (Rv3915)	ES	MAB_4942	ES	N-Acetylmuramoyl-L-alanine amidase CwlM
	ami3 (Rv3811)	NE	MAB_0168c	GA	Putative N-acetymuramoyl-L-alanine amidase
	ami4 (Rv3594)	NE	MAB_4807	NE	Bacteriophage protein

[a]Candidate genes were sourced and classified based on previous reports (49–51). The essentiality status of *M. tuberculosis* genes was referenced from DeJesus et al. (10). ES, essential; GD, growth defect when mutated; GA, growth advantage when mutated; NE, nonessential.

associated with peptidoglycan (PG) biosynthesis and remodeling, as previously identified and categorized (49–51). The essentiality comparisons of these 28 *M. abscessus* genes with 30 genes in *M. tuberculosis* H37Rv (10) indicated a few differences. For example, *Rv0024* and *rpfD* orthologs are absent in *M. abscessus* and *MAB_3167c* encoding a putative penicillin-binding protein is essential in *M. abscessus*, but its ortholog Rv2864c is nonessential in *M. tuberculosis* (Table 6). Similarly, *ripA* encoding an endopeptidase contains an essential domain in *M. tuberculosis*, but its ortholog *MAB_2728c* was classified as nonessential (Table 6). Remarkably, 11 *M. abscessus* genes were classified as GA genes compared to only one in *M. tuberculosis* (Table 6). The GA *M. abscessus* genes were found in each functional group, and their corresponding orthologs in *M. tuberculosis* are well characterized regarding their roles in PG biosynthesis (52–59). Among them, ldt_{Mab1} and ldt_{Mab2} were confirmed to encode L,D-transpeptidases in *M. abscessus* like their orthologs ldt_{Mt1} and ldt_{Mt2}, which are targets of cephalosporin and carbapenem β-lactams (55).

Essential *M. abscessus* genes without homologs in *M. tuberculosis* or *M. avium*. Most of the 43.6% of *M. abscessus* genes (2,145/4,920) with no significant similarity to either *M. tuberculosis* or *M. avium* are hypothetical genes. The majority were defined as nonessential, whereas 262 and 16 genes conferred a growth advantage and a growth defect, respectively, when interrupted. Four genes without TA sites could not be assessed. Many TetR family regulators, *whiB* regulators, drug-modifying enzymes, and efflux pumps belonged to this category, some of which are known to be involved in intrinsic drug resistance, such as *MAB_0591* (rifampin ADP-ribosyl transferase) (19), *MAB_4532c* (*eis2*, Gcn5-related *N*-acetyltransferase) (60, 61), and *MAB_4384* (TetR regulator) (62). Only seven essential *M. abscessus* genes lacked significant homology with *M. tuberculosis* or *M. avium* genes, most of which encode hypothetical proteins (Table 3). However, *MAB_3419* encodes NH_3-dependent NAD^+ synthetase (NadE) (63), which catalyzes the last step in *de novo* NAD^+ biosynthesis, and is a validated drug target in *M. tuberculosis* (64–66). Almost all external genes, including an 81-kb full-length prophage, 3 prophage-like elements, and 17 clusters of horizontally transferred genes from nonmycobacterial organisms described by Ripoll et al. (14), are not homologous to *M. tuberculosis* or *M. avium* genes and are dispensable for *in vitro* growth. However, two essential genes, *MAB_0222c* (putative DNA-binding protein) and *MAB_4828c* (unknown function) are located on distinct prophage-like elements (Table 3). Analysis of peptide sequences from 1,718 *M. abscessus* complex genomes deposited in GenBank using BLAST revealed homologs of *MAB_0222c* and *MAB_4828c* in only 23 and 307 genomes, respectively, and exclusively in *M. abscessus* subsp. *abscessus*. In addition, MAB_0222c showed the highest identity of 63% with a DNA-binding protein in *M. chelonae*, whereas MAB_4828c showed 72 and 75% identities to hypothetical proteins in *Mycobacteroides salmoniphilum* and *Mycobacteroides franklinii*, respectively. These strains were isolated from water and have caused fish and, rarely, human infections (67). Further comparative genomic analysis showed that 80% (3,940/4,920) of *M. abscessus* genes have homologs in *M. chelonae* type strain CCUG 47445 (68), including 96% (313/326) of essential *M. abscessus* genes (data not shown). Table S8 lists 13 essential genes without significant homologs in *M. chelonae* CCUG 47445. Interestingly, the MAB_0222c homolog was not identified in this strain.

Essentiality analysis of short ORFs and non-ORF genomic features. Short ORFs (sORFs) and non-ORF genomic features in *M. abscessus* ATCC 19977^T were first identified by Miranda-CasoLuengo et al. using RNA-seq/Ribo-seq and proteomics technologies (69). In all, 126 ribosomally protected sORFs were detected over the genome, 80% of which are ≤50 amino acids in length (69). Table 1 shows the essentiality assignments of sORFs, ncRNAs (noncoding RNAs), tRNAs (transfer RNAs), rRNAs (ribosomal RNAs), 5′ UTRs (5′ untranslated regions), promoter regions, and Rho-independent terminators. Essential genetic elements are shown in Table 7. Only 5 of 126 sORFs were defined as essential, although 17 without TA sites could not be assessed. Of 36 ncRNAs containing 55 to 404 nucleotides, 4 were classified as essential. Of 47 tRNA genes, 10 were classified as essential, while 2 without TA sites could not be assessed.

TABLE 7 Other essential genomic features of *M. abscessus* ATCC 19977[T]

sORF	ncRNA	tRNA	rRNA
MAB_5003	ncRNA_Mab1237c	MAB_t5001 (GAT,Ile)	MAB_r5051 (16sRNA,rrs)
MAB_5017c	ncRNA_Mab13471c	MAB_t5002 (TGC,Ala)	MAB_r5052 (23sRNA,rrl)
MAB_5034c	ncRNA_Mab1913	MAB_t5013 (TTC,Glu)	MAB_r5053 (5sRNA,rrf)
MAB_5035c	ncRNA_Mabr5052	MAB_t5014 (GTC,Asp)	
MAB_5050c		MAB_t5025c (TCC,Gly)	
		MAB_t5031c (GAG,Leu)	
		MAB_t5040c (CTG,Gln)	
		MAB_t5042c (CAT,Met)	
		MAB_t5044c (CAT,Met)	
		MAB_t5045c (GGT,Thr)	

Remarkably, 4 tRNA genes conferred a growth advantage when disrupted, and only 2 of 7 singleton tRNA genes (MAB_t5001 [Ile, GAT] and MAB_t5014 [Asp, GTC]) were essential. Interestingly, one or two copies of tRNA genes for transfer of Gln and Thr were essential in *M. abscessus*, *M. tuberculosis*, and *M. avium* (10, 11), suggesting that these two amino acids play crucial roles in mycobacterial physiology. As expected, the genes encoding 16S, 23S, and 5S rRNAs were essential in *M. abscessus* as in *M. tuberculosis* and *M. avium* (10, 11). 16S and 23S rRNAs are targets of amikacin and macrolides, respectively, which are first-line drugs recommended for treating *M. abscessus* infections (45). Most predicted promoter regions, 5′ UTRs and rho-independent terminators were nonessential. Detailed information on the essentiality analyses of sORFs and non-ORF genomic features is available in Data Set S1C.

Essentiality analysis of the plasmid. *M. abscessus* ATCC 19977[T] contains the 23-kb plasmid pMAB23, which is 99% identical to pMM23 from *Mycobacterium marinum* ATCC BAA-535 and harbors a putative mercury resistance operon (14, 70). All of the 22 annotated coding sequences and intergenic regions are dispensable for *in vitro* growth except *MAB_p16c* encoding putative replication protein RepA and the intergenic region upstream of *repA* (between *MAB_p17* and *MAB_p16c*), which were defined as GD when disrupted (see Data Set S1D).

DISCUSSION

Despite its increasing incidence as a cause of difficult-to-treat opportunistic infections, *M. abscessus* remains poorly studied, in part because it has been difficult to manipulate genetically. The availability of a comprehensive list of essential genes and other genomic features is a crucial step toward greater understanding of *M. abscessus* physiology and pathogenesis. To our knowledge, this is the first comprehensive analysis of the essentiality of *M. abscessus* genetic elements required for *in vitro* growth using fully saturated Tn mutant pools and deep sequencing.

Use of highly saturated Tn mutant libraries and an HMM increases the predictive accuracy of essentiality analyses, especially for classifying genomic features with fewer TA sites (10, 11). HMMs have proven to be a reliable statistical method for estimating different degrees of essentiality across the genome in an unbiased (non-gene-centered) way (10, 11, 16). However, it has been difficult to achieve high saturation using the Himar1 Tn in *M. abscessus*. To date, published reports studied *M. abscessus* Tn mutant pools containing only 6,000 to 8,000 unique Tn mutants (9, 36), far from the 91,240 TA sites in the genome of *M. abscessus* ATCC 19977[T]. Through systematic optimization of procedures for Himar1 Tn mutagenesis and use of triplicate Tn DNA libraries per Tn mutant pool, we increased the number of unique TA insertions to 67,518 to 71,167 per pool and achieved full saturation of detectable TA site insertions. The overall proportions of TA sites with or without observed Tn insertions (85.7 and 14.3%, respectively) and with a known nonpermissive motif (8.1%) are similar to those reported in *M. tuberculosis* and *M. avium* (10, 11). Our findings also indicate that, in

addition to the lethality of Tn insertions into essential genes and the restrictive effects of the nonpermissive motif (10), other unknown factors may restrict Himar1 insertion into certain TA sites. The Himar1 Tn is widely used in mutagenesis studies, but its utility and limitations in diverse mycobacterial genomes is understudied. Our optimization approach may provide a useful roadmap for generating more fully saturated Tn mutant pools in other mycobacteria.

The *M. abscessus* complex is a large and heterogeneous group of species (1, 71, 72) capable of causing opportunistic infections in any organ, but commonly in the lungs and skin and soft tissue (73). In-depth genomic analysis of *M. abscessus* indicates a non-conservative genome, in which the core genome is limited to 64.15% of the pan-genome, differing from the conservative pathogen *M. tuberculosis*, whose core genome represents 96.1% of the pan-genome (72). Despite *M. abscessus* diversity in genome size and content, our findings on the essentiality of genomic elements of *M. abscessus* ATCC 19977^{T} will shed light on other *M. abscessus* complex strains, especially many clinically relevant strains in the United States and Europe, since phylogenomic analyses place this type strain within the predominant clone observed in several global and national studies of clinical isolates (74). Most essential *M. abscessus* genes defined here are highly homologous to those identified in similar studies of *M. tuberculosis* and *M. avium*. These results provide a fundamental basis for utilizing available knowledge and approaches from *M. tuberculosis* and *M. avium* studies to promote research to address key knowledge gaps regarding *M. abscessus*. Our findings also highlight intriguing genomic differences that could be exploited for greater understanding of *M. abscessus* pathogenesis and development of new tools to treat and prevent *M. abscessus* infections.

Essential *M. abscessus* genes sharing significant homology with essential *M. tuberculosis* genes include validated targets for important anti-TB drugs, such as isoniazid (43), rifampin (17), ethambutol (44), moxifloxacin (37), and bedaquiline (20). However, these drugs are not effective against *M. abscessus* infections or, in the case of bedaquiline, require further study (21, 22, 38, 45). Thus, drugs developed and optimized against essential *M. tuberculosis* targets may not be useful against even highly homologous essential targets in *M. abscessus* due to interspecies differences in target protein structure or the presence or absence of enzymes that activate prodrugs like isoniazid or inactivate drugs, such as rifamycins, or other unique resistance mechanisms, such as efflux transporters (19, 47, 60–62, 75–78). Thus, developing new anti-*M. abscessus* drugs against drug targets validated in TB should be an effective approach, but programs focused specifically on *M. abscessus* are needed to deliver optimized drugs that exploit interspecies differences in structure-activity relationships (SAR) and intrinsic resistance mechanisms. For example, our approach predicted MmpL3 (MAB_4508) to be essential in *M. abscessus*, as in *M. tuberculosis*. This flippase required for translocating mycolate precursors to the cell envelope was successfully targeted first in *M. tuberculosis* by a series of indole-2-carboxamide inhibitors but subsequent evolution of this series and others based on unique SAR delivered compounds with superior *in vitro* and *in vivo* activity against *M. abscessus* (46, 79–82). Glutamine synthase GlnA1 (MAB_1933c) is predicted to be essential in *M. abscessus* and may represent a more novel drug target and virulence factor. The attenuation of an *M. tuberculosis* glnA1 deletion mutant during glutamine auxotrophy and in guinea pigs and mice is encouraging in this regard (83, 84), especially since glutamine is not readily available in CF sputum, an important niche for *M. abscessus* (85). Furthermore, genetic or chemical disruption of GlnA1 increases vulnerability to bedaquiline in *M. tuberculosis* (27), suggesting that a MAB_1933c inhibitor could synergize with diarylquinolines against *M. abscessus*.

Genes essential in *M. abscessus* but not in *M. tuberculosis* could also be more effectively exploited as drug targets in *M. abscessus*. *MAB_3090c* encodes the dihydrofolate reductase (DHFR) DfrA, a conserved enzyme in the folate biosynthesis pathway (86). Although DHFR inhibitors are effective anti-proliferative drug targets for treating a variety of malignancies and autoimmune and infectious diseases (86, 87), they have not

proven very effective against *M. tuberculosis* (88). However, DHFR is only conditionally essential in *M. tuberculosis* (10) and is not particularly vulnerable because its loss can be compensated by upregulation of a second DHFR enzyme, Rv2671, and ThyX (89, 90). Whether the nonessential Rv2671 ortholog MAB_2976 and ThyX (Rv2754c) ortholog MAB_3085c that showed a growth advantage after disruption can compensate for the loss of DfrA in *M. abscessus* may determine the value of this target in *M. abscessus*.

Genes essential in *M. abscessus* that have limited or no homology with genes in *M. tuberculosis* may represent new and more specific drug targets. Of particular interest is *MAB_3419*, a putative ammonia-dependent NAD synthetase (NadE) that catalyzes the final step in NAD^+ biosynthesis. NAD^+ is an essential cofactor that mycobacteria synthesize either *de novo* from aspartate or from nicotinamide/nicotinic acid scavenged from the environment. Both pathways utilize NadE. Interestingly, *MAB_3419* is predicted to be a single-domain NAD^+ synthetase that uses ammonia as a nitrogen source, with >90% protein sequence homology with NadE in *Mycobacterium chelonae* and a few other rapidly growing mycobacteria but limited homology with the *M. tuberculosis* and *Mycobacterium smegmatis* enzymes, which are glutamine-dependent NAD+ synthetases comprised of a C-terminal NAD^+ synthetase domain fused with an N-terminal glutaminase domain. NadE is a genetically and chemically validated drug target in *M. tuberculosis* l (91–93), but the described inhibitors bind to sites not present in MAB_3419, indicating a different chemical route is required to target *M. abscessus* NadE.

The mycobacterial cell wall is an essential structure for growth and virulence. Comprised of three distinct layers (PG, arabinogalactan, and mycolic acids), it is an attractive target for antimycobacterial antibiotics (48). Unlike in TB, PG synthesis inhibitors, i.e., imipenem and cefoxitin are already first-line drugs for *M. abscessus* infections. PG requires constant expansion, remodeling and recycling during bacterial growth and division (94). We identified interesting differences in the essentiality of genes associated with PG metabolism between *M. abscessus* and *M. tuberculosis*. Although the growth-advantaged phenotypes of Tn insertions in 11 PG-associated *M. abscessus* genes need to be confirmed, we speculate that *M. abscessus* has evolved interaction networks that differ from those in *M. tuberculosis* and may confer a greater ability to compensate for disruption of certain PG-synthesizing enzymes to ensure cell wall integrity and greater adaptability to changing environmental conditions.

Approximately 5 to 6% of genes in the *M. abscessus* genome were likely acquired through horizontal gene transfer from other organisms (14). Among them, only two genes from prophage-like elements (*MAB_0222c* and *MAB_4828c*) are defined as essential for *in vitro* growth. Horizontal gene transfer preferentially occurs between specific groups of organisms that share ancestry or habitat, presumably under evolutionary pressure (95, 96). The genes' homology to *M. chelonae* and to *M. salmoniphilum* and *M. franklinii*, respectively, suggests horizontal gene transfer in a shared habitat, perhaps in water (67). Since the genes are found in the more pathogenic *M. abscessus* subsp. *abscessus* but not in *M. abscessus* subsp. *massiliense* or *M. abscessus* subsp. *bolletii*, one wonders whether their acquisition improves pathogenicity in addition to adaptation and survival in the environment.

We evaluated the essentiality of 126 previously identified sORFs (69). Interestingly, essential sORFs also showed significant responses to stress conditions in previous RNA-seq studies. For instance, *MAB_5003*, *MAB_5034c*, and *MAB_5035c* are significantly upregulated, while *MAB_5050c* is downregulated in artificial sputum, and increased expression of *MAB_5035c* was detected in response to kanamycin (69). *MAB_5050c* is located at the leader region of essential gene *MAB_3798c* encoding 30S ribosomal protein S8 (RpsH) that directly binds to 16S rRNA to assemble the 30S subunit of the ribosome for protein synthesis (97). RpsH also plays a critical role in selectively inhibiting synthesis of ribosomal proteins whose genes are in the same *spc* operon as its own in response to changing environmental conditions (98). Although any functional association between MAB_5050c and RpsH requires further investigation, downregulation of

MAB_5050c may play some role in regulating protein synthesis under stress. tRNAs play a central role in protein translation and thus are potential targets for new antibiotics that inhibit attachment of amino acids onto corresponding tRNAs. Most *M. abscessus* tRNA genes are dispensable for *in vitro* growth, as in *M. tuberculosis* and *M. avium* (10, 11). Only two of seven singleton tRNAs were defined as essential, similar to findings in *Saccharomyces cerevisiae*, in which four of six singleton tRNA genes are essential and most tRNA deletions do not cause altered growth phenotypes in rich medium (99), presumably because they are compensated by members of the same or different anti-codon families in many conditions (99).

We defined essential genes under optimal *in vitro* growth conditions using a reliable and comprehensive approach. However, further confirmation of essentiality may still be necessary. Genes not required for *in vitro* growth could become conditionally essential when *M. abscessus* encounters certain stresses during infection, including in the nutritionally aberrant environments of cystic fibrosis airway mucus, biofilms, in phagocytes, and necrotic pyogranulomatous lesions. For example, although genes comprising the ESX-3 and ESX-4 loci are nonessential under conditions in this study, *M. abscessus* ESX-3 plays an important role in pathogenesis (100), and *M. abscessus* ESX-4 genes are required for intracellular survival (36). Nevertheless, differences in essentiality and homology between these genes in *M. abscessus* and *M. tuberculosis* may indicate differences in function, which deserves further study. Using saturated *M. abscessus* Tn mutant libraries to identify new virulence factors and potential drug targets under clinically relevant conditions is under investigation.

MATERIALS AND METHODS

Bacterial strain, bacteriophage, and media. *M. abscessus* ATCC 19977^T type strain was purchased from the American Type Culture Collection (ATCC). *Mycobacterium smegmatis* mc^{2}155 and bacteriophage ΦmycomarT7 came from stocks described previously (101). Unless stated otherwise, Middlebrook 7H9 broth base (Difco, BD) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) complex (BD), 0.5% glycerol, and 0.05% Tween 80 (Sigma-Aldrich) (7H9 broth) were used for cultivation. 7H11 agar (Difco, BD) containing 10% OADC, 0.5% glycerol, and 0.1% Tween 80 were used to select Tn mutants (7H11 agar). Top agar containing 0.5 g of 7H9 broth base, 0.7 g of Bacto agar (Difco, BD), and 0.5 ml of glycerol in 100 ml of distilled water was prepared and poured on 7H11 agar plates when needed for phage studies.

Construction of Himar1 Tn mutant pools. A protocol for constructing a saturated Himar1 Tn mutant pool was carefully optimized based on the literatures (11, 102). Detailed information was provided in the supplemental material.

Preparation of DNA libraries of Tn mutant pools. DNA libraries of Tn mutant pools were prepared as previously described with modification (11, 102). Detailed methods are provided in Text S1 in the supplemental material.

Deep sequencing of Tn insertions and analysis of sequencing data. Thirty Tn mutant DNA libraries representing triplicate samples from each of 10 Tn mutant pools were sequenced on an Illumina HiSeq instrument, collecting 18 (10.4 to 31.5) million 150-bp paired-end reads per sample. The reads were filtered by Trimmomatic v0.39 (103) and processed using TPP in TRANSIT, which counts reads mapping to each TA dinucleotide site against the published *M. abscessus* ATCC 19977^T (accession number CU458896) genome sequence (after eliminating reads sharing the same template barcode) (15). A Hidden Markov Model (HMM) offered by TRANSIT was used to assign the most probable state of essentiality of the sequence of TA sites based on the read count at the site and the distribution over the surrounding sites (15, 16). It parses a genome into contiguous regions belonging to one of four essentiality states—essential (ES), nonessential (NE), growth defect when mutated (GD), or growth advantage when mutated (GA), based on local insertion density and mean value of nonempty read counts at TA sites (with ES being near 0, NE being near the mean, and GD and GA being approximately 1/10 and 5 times the mean, respectively). To search for TA site motifs that are less permissive for Himar1 Tn insertion in the *M. abscessus* genome, all TA sites without insertions but excluding TA sites from essential gene regions or regulatory regions which may be under selection pressure were selected as a putative nonpermissive set (~6,000 TA sites). The TA site regions with the top 25% read counts of Himar1 Tn insertions were then chosen as a putative permissive set. The nucleotides surrounding TA sites were compared between the two sets.

Ortholog analysis was performed among all annotated genes and the subset of predicted essential genes of *M. abscessus* ATCC 19977^T, *M. tuberculosis* H37Rv, and *M. avium* MAC109 (10, 11) using orthovenn2 diagram analysis (excluding genes encoding proteins containing less than 20 amino acids) (104). Homology searches were performed between the genomes of *M. abscessus* ATCC 19977^T and *M. chelonae* type strain CCUG 47445 (68) using the same method. Other genomic features, including new sORFs and non-ORF genomic elements, were identified based on published information (69). Promoter regions were defined based on a set of 2,653 transcriptional start sites (TSSs) defined previously (69). A region

around each TSS (bp −150 to +70) was used to determine the promoter region as described previously (10). Lastly, Rho-independent transcription terminators were predicted using the ARNold online tool (http://rssf.i2bc.paris-saclay.fr/toolbox/arnold/) (105).

Data availability. The raw data are deposited in NCBI SRA database under BioSample accession numbers SAMN16825978 to SAMN16826007.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

DATA SET S1, XLS file, 9.1 MB.
TEXT S1, DOCX file, 0.04 MB.
TABLE S1, DOCX file, 0.04 MB.
TABLE S2, DOCX file, 0.1 MB.
TABLE S3, DOCX file, 0.03 MB.
TABLE S4, DOCX file, 0.04 MB.
TABLE S5, DOCX file, 0.03 MB.
TABLE S6, DOCX file, 0.04 MB.
TABLE S7, DOCX file, 0.03 MB.
TABLE S8, DOCX file, 0.02 MB.

REFERENCES

1. Brown-Elliott BA, Wallace RJ, Jr. 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev 15:716–746. https://doi.org/10.1128/cmr.15.4.716-746.2002.
2. Johansen MD, Herrmann JL, Kremer L. 2020. Non-tuberculous mycobacteria and the rise of Mycobacterium abscessus. Nat Rev Microbiol 18:392–407. https://doi.org/10.1038/s41579-020-0331-1.
3. Newman MI, Camberos AE, Clynes ND, Ascherman JA. 2005. Outbreak of atypical mycobacteria infections in U.S. patients traveling abroad for cosmetic surgery. Plast Reconstr Surg 115:964–965. https://doi.org/10.1097/01.prs.0000153818.99552.cc.
4. Bryant JM, Grogono DM, Rodriguez-Rincon D, Everall I, Brown KP, Moreno P, Verma D, Hill E, Drijkoningen J, Gilligan P, Esther CR, Noone PG, Giddings O, Bell SC, Thomson R, Wainwright CE, Coulter C, Pandey S, Wood ME, Stockwell RE, Ramsay KA, Sherrard LJ, Kidd TJ, et al. 2016. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. Science 354:751–757. https://doi.org/10.1126/science.aaf8156.
5. Degiacomi G, Sammartino JC, Chiarelli LR, Riabova O, Makarov V, Pasca MR. 2019. Mycobacterium abscessus, an emerging and worrisome pathogen among cystic fibrosis patients. Int J Mol Sci 20 https://doi.org/10.3390/ijms20235868.
6. Lopeman RC, Harrison J, Desai M, Cox JAG. 2019. Mycobacterium abscessus: environmental bacterium turned clinical nightmare. Microorganisms 7:90. https://doi.org/10.3390/microorganisms7030090.
7. Cortes M, Singh AK, Reyrat JM, Gaillard JL, Nassif X, Herrmann JL. 2011. Conditional gene expression in Mycobacterium abscessus. PLoS One 6:e29306. https://doi.org/10.1371/journal.pone.0029306.
8. Medjahed H, Reyrat JM. 2009. Construction of Mycobacterium abscessus defined glycopeptidolipid mutants: comparison of genetic tools. Appl Environ Microbiol 75:1331–1338. https://doi.org/10.1128/AEM.01914-08.
9. Foreman M, Gershoni M, Barkan D. 2020. A simplified and efficient method for Himar-1 transposon sequencing in bacteria, demonstrated by creation and analysis of a saturated transposon-mutant library in

Mycobacterium abscessus. mSystems 5:e00976-20. https://doi.org/10.1128/mSystems.00976-20.
10. DeJesus MA, Gerrick ER, Xu W, Park SW, Long JE, Boutte CC, Rubin EJ, Schnappinger D, Ehrt S, Fortune SM, Sassetti CM, Ioerger TR. 2017. Comprehensive essentiality analysis of the Mycobacterium tuberculosis genome via saturating transposon mutagenesis. mBio 8:e02133-16. https://doi.org/10.1128/mBio.02133-16.
11. Matern WM, Jenquin RL, Bader JS, Karakousis PC. 2020. Identifying the essential genes of Mycobacterium avium subsp. hominissuis with Tn-Seq using a rank-based filter procedure. Sci Rep 10:1095. https://doi.org/10.1038/s41598-020-57845-7.
12. Dragset MS, Ioerger TR, Loevenich M, Haug M, Sivakumar N, Marstad A, Cardona PJ, Klinkenberg G, Rubin EJ, Steigedal M, Flo TH. 2019. Global assessment of Mycobacterium avium subsp. hominissuis genetic requirement for growth and virulence. mSystems 4:e00402-19. https://doi.org/10.1128/mSystems.00402-19.
13. Minato Y, Gohl DM, Thiede JM, Chacón JM, Harcombe WR, Maruyama F, Baughn AD. 2019. Genomewide assessment of Mycobacterium tuberculosis conditionally essential metabolic pathways. mSystems 4:e00070-19. https://doi.org/10.1128/mSystems.00070-19.
14. Ripoll F, Pasek S, Schenowitz C, Dossat C, Barbe V, Rottman M, Macheras E, Heym B, Herrmann J-L, Daffé M, Brosch R, Risler J-L, Gaillard J-L. 2009. Non mycobacterial virulence genes in the genome of the emerging pathogen Mycobacterium abscessus. PLoS One 4:e5660. https://doi.org/10.1371/journal.pone.0005660.
15. DeJesus MA, Ambadipudi C, Baker R, Sassetti C, Ioerger TR. 2015. TRANSIT: a software tool for Himar1 TnSeq analysis. PLoS Comput Biol 11:e1004401. https://doi.org/10.1371/journal.pcbi.1004401.
16. DeJesus MA, Ioerger TR. 2013. A Hidden Markov Model for identifying essential and growth-defect regions in bacterial genomes from transposon insertion sequencing data. BMC Bioinformatics 14:303. https://doi.org/10.1186/1471-2105-14-303.
17. Rosenthal IM, Tasneen R, Peloquin CA, Zhang M, Almeida D, Mdluli KE, Karakousis PC, Grosset JH, Nuermberger EL. 2012. Dose-ranging

comparison of rifampin and rifapentine in two pathologically distinct murine models of tuberculosis. Antimicrob Agents Chemother 56:4331–4340. https://doi.org/10.1128/AAC.00912-12.

18. Sha W, Weng XH, Xiao HP, He GJ. [2003. Investigation of drug-resistance to rifampin and *rpoB* gene sequence analysis of *Mycobacterium abscessus*. Chin J Tuberc Respir Dis 26:544–547.

19. Rominski A, Roditscheff A, Selchow P, Bottger EC, Sander P. 2017. Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591. J Antimicrob Chemother 72:376–384. https://doi.org/10.1093/jac/dkw466.

20. Sarathy JP, Gruber G, Dick T. 2019. Re-understanding the mechanisms of action of the anti-mycobacterial drug bedaquiline. Antibiotics (Basel) 8:261. https://doi.org/10.3390/antibiotics8040261.

21. Dupont C, Viljoen A, Thomas S, Roquet-Banères F, Herrmann J-L, Pethe K, Kremer L. 2017. Bedaquiline inhibits the ATP synthase in *Mycobacterium abscessus* and is effective in infected zebrafish. Antimicrob Agents Chemother 61:e01225-17. https://doi.org/10.1128/AAC.01225-17.

22. Brown-Elliott BA, Wallace RJ, Jr. 2019. *In vitro* susceptibility testing of bedaquiline against *Mycobacterium abscessus* complex. Antimicrob Agents Chemother 63:e01919-18. https://doi.org/10.1128/AAC.01919-18.

23. Alexander DC, Vasireddy R, Vasireddy S, Philley JV, Brown-Elliott BA, Perry BJ, Griffith DE, Benwill JL, Cameron ADS, Wallace RJ. 2017. Emergence of *mmpT5* variants during bedaquiline treatment of *Mycobacterium intracellulare* lung disease. J Clin Microbiol 55:574–584. https://doi.org/10.1128/JCM.02087-16.

24. Lounis N, Gevers T, Van den Berg J, Vranckx L, Andries K. 2009. ATP synthase inhibition of *Mycobacterium avium* is not bactericidal. Antimicrob Agents Chemother 53:4927–4929. https://doi.org/10.1128/AAC.00689-09.

25. Krajewski WW, Jones TA, Mowbray SL. 2005. Structure of *Mycobacterium tuberculosis* glutamine synthetase in complex with a transition-state mimic provides functional insights. Proc Natl Acad Sci U S A 102:10499–10504. https://doi.org/10.1073/pnas.0502248102.

26. Reitzer L. 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. Annu Rev Microbiol 57:155–176. https://doi.org/10.1146/annurev.micro.57.030502.090820.

27. Wang Z, Soni V, Marriner G, Kaneko T, Boshoff HIM, Barry CE, Rhee KY, III. 2019. Mode-of-action profiling reveals glutamine synthetase as a collateral metabolic vulnerability of *Mycobacterium tuberculosis* to bedaquiline. Proc Natl Acad Sci U S A 116:19646–19651. https://doi.org/10.1073/pnas.1907946116.

28. Bretl DJ, Bigley TM, Terhune SS, Zahrt TC. 2014. The MprB extracytoplasmic domain negatively regulates activation of the *Mycobacterium tuberculosis* MprAB two-component system. J Bacteriol 196:391–406. https://doi.org/10.1128/JB.01064-13.

29. Miller RA, Britigan BE. 1997. Role of oxidants in microbial pathophysiology. Clin Microbiol Rev 10:1–18. https://doi.org/10.1128/CMR.10.1.1-18.1997.

30. Mayer BK, Falkinham JO, III. 1986. Superoxide dismutase activity of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Infect Immun 53:631–635. https://doi.org/10.1128/IAI.53.3.631-635.1986.

31. Liao D, Fan Q, Bao L. 2013. The role of superoxide dismutase in the survival of *Mycobacterium tuberculosis* in macrophages. Jpn J Infect Dis 66:480–488. https://doi.org/10.7883/yoken.66.480.

32. Personne Y, Parish T. 2014. *Mycobacterium tuberculosis* possesses an unusual tmRNA rescue system. Tuberculosis (Edinb) 94:34–42. https://doi.org/10.1016/j.tube.2013.09.007.

33. Karzai AW, Roche ED, Sauer RT. 2000. The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. Nat Struct Biol 7:449–455. https://doi.org/10.1038/75843.

34. Bitter W, Houben ENG, Bottai D, Brodin P, Brown EJ, Cox JS, Derbyshire K, Fortune SM, Gao L-Y, Liu J, Gey van Pittius NC, Pym AS, Rubin EJ, Sherman DR, Cole ST, Brosch R. 2009. Systematic genetic nomenclature for type VII secretion systems. PLoS Pathog 5:e1000507. https://doi.org/10.1371/journal.ppat.1000507.

35. Dumas E, Christina Boritsch E, Vandenbogaert M, Rodríguez de la Vega RC, Thiberge J-M, Caro V, Gaillard J-L, Heym B, Girard-Misguich F, Brosch R, Sapriel G. 2016. Mycobacterial pan-genome analysis suggests important role of plasmids in the radiation of type VII secretion systems. Genome Biol Evol 8:387–402. https://doi.org/10.1093/gbe/evw001.

36. Laencina L, Dubois V, Le Moigne V, Viljoen A, Majlessi L, Pritchard J, Bernut A, Piel L, Roux A-L, Gaillard J-L, Lombard B, Loew D, Rubin EJ, Brosch R, Kremer L, Herrmann J-L, Girard-Misguich F. 2018. Identification of genes required for *Mycobacterium abscessus* growth *in vivo* with a prominent role

of the ESX-4 locus. Proc Natl Acad Sci U S A 115:E1002–E1011. https://doi.org/10.1073/pnas.1713195115.

37. Nagaraja V, Godbole AA, Henderson SR, Maxwell A. 2017. DNA topoisomerase I and DNA gyrase as targets for TB therapy. Drug Discov Today 22:510–518. https://doi.org/10.1016/j.drudis.2016.11.006.

38. Nie WJ, Xie ZY, Gao S, Teng TL, Zhou WQ, Shang YY, Jing W, Shi WH, Wang QF, Huang XR, Cai BY, Wang J, Wang J, Guo R, Ge QP, Nie LH, Han XQ, Du YD, Chu NH. 2020. Efficacy of moxifloxacin against *Mycobacterium abscessus* in zebrafish model *in vivo*. Biomed Environ Sci 33:350–358. https://doi.org/10.3967/bes2020.047.

39. Choi GE, Min KN, Won CJ, Jeon K, Shin SJ, Koh WJ. 2012. Activities of moxifloxacin in combination with macrolides against clinical isolates of *Mycobacterium abscessus* and *Mycobacterium massiliense*. Antimicrob Agents Chemother 56:3549–3555. https://doi.org/10.1128/AAC.00685-12.

40. Locher CP, Jones SM, Hanzelka BL, Perola E, Shoen CM, Cynamon MH, Ngwane AH, Wiid IJ, van Helden PD, Betoudji F, Nuermberger EL, Thomson JA. 2015. A novel inhibitor of gyrase B is a potent drug candidate for treatment of tuberculosis and nontuberculosis mycobacterial infections. Antimicrob Agents Chemother 59:1455–1465. https://doi.org/10.1128/AAC.04347-14.

41. Brown-Elliott BA, Rubio A, Wallace RJ, Jr. 2018. *In vitro* susceptibility testing of a novel benzimidazole, SPR719, against nontuberculous mycobacteria. Antimicrob Agents Chemother 62:e01503-18. https://doi.org/10.1128/AAC.01503-18.

42. Stokes SS, Vemula R, Pucci MJ. 2020. Advancement of GyrB inhibitors for treatment of infections caused by *Mycobacterium tuberculosis* and nontuberculous mycobacteria. ACS Infect Dis 6:1323–1331. https://doi.org/10.1021/acsinfecdis.0c00025.

43. Amin AG, Goude R, Shi L, Zhang J, Chatterjee D, Parish T. 2008. EmbA is an essential arabinosyltransferase in *Mycobacterium tuberculosis*. Microbiology (Reading) 154:240–248. https://doi.org/10.1099/mic.0.2007/012153-0.

44. Holas O, Ondrejcek P, Dolezal M. 2015. *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase inhibitors as potential antituberculotics: development in the past decade. J Enzyme Inhib Med Chem J 30:629–648. https://doi.org/10.3109/14756366.2014.959512.

45. Gopalaswamy R, Shanmugam S, Mondal R, Subbian S. 2020. Of tuberculosis and non-tuberculous mycobacterial infections: a comparative analysis of epidemiology, diagnosis and treatment. J Biomed Sci 27:74. https://doi.org/10.1186/s12929-020-00667-6.

46. Dupont C, Viljoen A, Dubar F, Blaise M, Bernut A, Pawlik A, Bouchier C, Brosch R, Guérardel Y, Lelièvre J, Ballell L, Herrmann J-L, Biot C, Kremer L. 2016. A new piperidinol derivative targeting mycolic acid transport in *Mycobacterium abscessus*. Mol Microbiol 101:515–529. https://doi.org/10.1111/mmi.13406.

47. Soroka D, Dubée V, Soulier-Escrihuela O, Cuinet G, Hugonnet J-E, Gutmann L, Mainardi J-L, Arthur M. 2014. Characterization of broad-spectrum *Mycobacterium abscessus* class A beta-lactamase. J Antimicrob Chemother 69:691–696. https://doi.org/10.1093/jac/dkt410.

48. Alderwick LJ, Birch HL, Mishra AK, Eggeling L, Besra GS. 2007. Structure, function and biosynthesis of the *Mycobacterium tuberculosis* cell wall: arabinogalactan and lipoarabinomannan assembly with a view to discovering new drug targets. Biochem Soc Trans 35:1325–1328. https://doi.org/10.1042/BST0351325.

49. Machowski EE, Senzani S, Ealand C, Kana BD. 2014. Comparative genomics for mycobacterial peptidoglycan remodelling enzymes reveals extensive genetic multiplicity. BMC Microbiol 14:75. https://doi.org/10.1186/1471-2180-14-75.

50. Arora D, Chawla Y, Malakar B, Singh A, Nandicoori VK. 2018. The transpeptidase PbpA and noncanonical transglycosylase RodA of *Mycobacterium tuberculosis* play important roles in regulating bacterial cell lengths. J Biol Chem 293:6497–6516. https://doi.org/10.1074/jbc.M117.811190.

51. Datta P, Dasgupta A, Singh AK, Mukherjee P, Kundu M, Basu J. 2006. Interaction between FtsW and penicillin-binding protein 3 (PBP3) directs PBP3 to mid-cell, controls cell septation and mediates the formation of a trimeric complex involving FtsZ, FtsW and PBP3 in mycobacteria. Mol Microbiol 62:1655–1673. https://doi.org/10.1111/j.1365-2958.2006.05491.x.

52. Kieser KJ, Baranowski C, Chao MC, Long JE, Sassetti CM, Waldor MK, Sacchettini JC, Ioerger TR, Rubin EJ. 2015. Peptidoglycan synthesis in *Mycobacterium tuberculosis* is organized into networks with varying drug susceptibility. Proc Natl Acad Sci U S A 112:13087–13092. https://doi.org/10.1073/pnas.1514135112.

53. Pandey SD, Pal S, Kumar NG, Bansal A, Mallick S, Ghosh AS. 2018. Two DD-carboxypeptidases from *Mycobacterium smegmatis* affect cell surface properties through regulation of peptidoglycan cross-linking and

54. Kana BD, Gordhan BG, Downing KJ, Sung N, Vostroktunova G, Machowski EE, Tsenova L, Young M, Kaprelyants A, Kaplan G, Mizrahi V. 2008. The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. Mol Microbiol 67:672–684. https://doi.org/10.1111/j.1365-2958.2007.06078.x.

55. Kumar P, Chauhan V, Silva JRA, Lameira J, d'Andrea FB, Li S-G, Ginell SL, Freundlich JS, Alves CN, Bailey S, Cohen KA, Lamichhane G. 2017. *Mycobacterium abscessus* L,D-transpeptidases are susceptible to inactivation by carbapenems and cephalosporins but not penicillins. Antimicrob Agents Chemother 61:e00866-17. https://doi.org/10.1128/AAC.00866-17.

56. Correale S, Ruggiero A, Capparelli R, Pedone E, Berisio R. 2013. Structures of free and inhibited forms of the L,D-transpeptidase LdtMt1 from *Mycobacterium tuberculosis*. Acta Crystallogr D Biol Crystallogr 69:1697–1706. https://doi.org/10.1107/S0907444913013085.

57. Gupta R, Lavollay M, Mainardi JL, Arthur M, Bishai WR, Lamichhane G. 2010. The *Mycobacterium tuberculosis* protein LdtMt2 is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. Nat Med 16:466–469. https://doi.org/10.1038/nm.2120.

58. Senzani S, Li D, Bhaskar A, Ealand C, Chang J, Rimal B, Liu C, Joon Kim S, Dhar N, Kana B. 2017. An amidase_3 domain-containing *N*-acetylmuramyl-L-alanine amidase is required for mycobacterial cell division. Sci Rep 7:1140. https://doi.org/10.1038/s41598-017-01184-7.

59. Kumar A, Kumar S, Kumar D, Mishra A, Dewangan RP, Shrivastava P, Ramachandran S, Taneja B. 2013. The structure of Rv3717 reveals a novel amidase from *Mycobacterium tuberculosis*. Acta Crystallogr D Biol Crystallogr 69:2543–2554. https://doi.org/10.1107/S0907444913026371.

60. Wu M, Li B, Guo Q, Xu L, Zou Y, Zhang Y, Zhan M, Xu B, Ye M, Yu F, Zhang Z, Chu H. 2019. Detection and molecular characterization of amikacin-resistant *Mycobacterium abscessus* isolated from patients with pulmonary disease. J Glob Antimicrob Resist 19:188–191. https://doi.org/10.1016/j.jgar.2019.05.016.

61. Pryjma M, Burian J, Kuchinski K, Thompson CJ. 2017. Antagonism between front-line antibiotics clarithromycin and amikacin in the treatment of *Mycobacterium abscessus* infections is mediated by the *whiB7* gene. Antimicrob Agents Chemother 61:e01353-17. https://doi.org/10.1128/AAC.01353-17.

62. Richard M, Gutierrez AV, Viljoen AJ, Ghigo E, Blaise M, Kremer L. 2018. Mechanistic and structural insights into the unique TetR-dependent regulation of a drug efflux pump in *Mycobacterium abscessus*. Front Microbiol 9:649. https://doi.org/10.3389/fmicb.2018.00649.

63. Santos ARS, Gerhardt ECM, Moure VR, Pedrosa FO, Souza EM, Diamanti R, Högbom M, Huergo LF. 2018. Kinetics and structural features of dimeric glutamine-dependent bacterial NAD$^+$ synthetases suggest evolutionary adaptation to available metabolites. J Biol Chem 293:7397–7407. https://doi.org/10.1074/jbc.RA118.002241.

64. Rizzi M, Schindelin H. 2002. Structural biology of enzymes involved in NAD and molybdenum cofactor biosynthesis. Curr Opin Struct Biol 12:709–720. https://doi.org/10.1016/S0959-440X(02)00385-8.

65. Zalkin H, Smith JL. 1998. Enzymes utilizing glutamine as an amide donor. Adv Enzymol Relat Areas Mol Biol 72:87–144. https://doi.org/10.1002/9780470123188.ch4.

66. Bellinzoni M, Buroni S, Pasca MR, Guglierame P, Arcesi F, De Rossi E, Riccardi G. 2005. Glutamine amidotransferase activity of NAD$^+$ synthetase from *Mycobacterium tuberculosis* depends on an amino-terminal nitrilase domain. Res Microbiol 156:173–177. https://doi.org/10.1016/j.resmic.2004.08.011.

67. Behra PRK, Das S, Pettersson BMF, Shirreff L, DuCote T, Jacobsson K-G, Ennis DG, Kirsebom LA. 2019. Extended insight into the *Mycobacterium chelonae-abscessus* complex through whole-genome sequencing of *Mycobacterium salmoniphilum* outbreak and *Mycobacterium salmoniphilum*-like strains. Sci Rep 9:4603. https://doi.org/10.1038/s41598-019-40922-x.

68. Jaén-Luchoro D, Salvà-Serra F, Aliaga-Lozano F, Seguí C, Busquets A, Ramírez A, Ruíz M, Gomila M, Lalucat J, Bennasar-Figueras A. 2016. Complete genome sequence of *Mycobacterium chelonae* type strain CCUG 47445, a rapidly growing species of nontuberculous mycobacteria. Genome Announc 4. https://doi.org/10.1128/genomeA.00550-16.

69. Miranda-CasoLuengo AA, Staunton PM, Dinan AM, Lohan AJ, Loftus BJ. 2016. Functional characterization of the *Mycobacterium abscessus* genome coupled with condition specific transcriptomics reveals conserved molecular strategies for host adaptation and persistence. BMC Genomics 17:553. https://doi.org/10.1186/s12864-016-2868-y.

70. Kim B-J, Cha G-Y, Kim B-R, Kook Y-H, Kim B-J. 2019. Insights from the genome sequence of *Mycobacterium paragordonae*, a potential novel live vaccine for preventing mycobacterial infections: the putative role of type VII secretion systems for an intracellular lifestyle within free-living environmental predators. Front Microbiol 10:1524. https://doi.org/10.3389/fmicb.2019.01524.

71. Chhotaray C, Wang S, Tan Y, Ali A, Shehroz M, Fang C, Liu Y, Lu Z, Cai X, Hameed HMA, Islam MM, Surineni G, Tan S, Liu J, Zhang T. 2020. Comparative analysis of whole-genome and methylome profiles of a smooth and a rough *Mycobacterium abscessus* clinical strain. G3 (Bethesda) 10:13–22. https://doi.org/10.1534/g3.119.400737.

72. Sassi M, Drancourt M. 2014. Genome analysis reveals three genomospecies in *Mycobacterium abscessus*. BMC Genomics 15:359. https://doi.org/10.1186/1471-2164-15-359.

73. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Winthrop K, Infectious Disease Society of America. 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 175:367–416. https://doi.org/10.1164/rccm.200604-571ST.

74. Hasan NA, Davidson RM, Epperson LE, Kammlade SM, Rodger RR, Levin AR. 2019. Population genomics of nontuberculous mycobacteria recovered from United States cystic fibrosis patients. bioRxiv 663559. https://doi.org/10.1101/663559.

75. Nash KA, Brown-Elliott BA, Wallace RJ, Jr. 2009. A novel gene, *erm*(41), confers inducible macrolide resistance to clinical isolates of *Mycobacterium abscessus* but is absent from *Mycobacterium chelonae*. Antimicrob Agents Chemother 53:1367–1376. https://doi.org/10.1128/AAC.01275-08.

76. Quan S, Imai T, Mikami Y, Yazawa K, Dabbs ER, Morisaki N, Iwasaki S, Hashimoto Y, Furihata K. 1999. ADP-ribosylation as an intermediate step in inactivation of rifampin by a mycobacterial gene. Antimicrob Agents Chemother 43:181–184. https://doi.org/10.1128/AAC.43.1.181.

77. Reingewertz TH, Meyer T, McIntosh F, Sullivan J, Meir M, Chang Y-F, Behr MA, Barkan D. 2019. Differential sensitivity of mycobacteria to isoniazid is related to differences in KatG-mediated enzymatic activation of the drug. Antimicrob Agents Chemother 64:e01899-19. https://doi.org/10.1128/AAC.01899-19.

78. Gutierrez AV, Richard M, Roquet-Baneres F, Viljoen A, Kremer L. 2019. The TetR family transcription factor MAB_2299c regulates the expression of two distinct MmpS-MmpL efflux pumps involved in cross-resistance to clofazimine and bedaquiline in *Mycobacterium abscessus*. Antimicrob Agents Chemother 63:e01000-19. https://doi.org/10.1128/AAC.01000-19.

79. Franz ND, Belardinelli JM, Kaminski AM, Dunn LC, Calado Nogueira de Moura V, Blaha MA, Truong DD, Li W, Jackson M, North EJ. 2017. Design, synthesis and evaluation of indole-2-carboxamides with pan anti-mycobacterial activity. Bioorg Med Chem 25:3746–3755. https://doi.org/10.1016/j.bmc.2017.05.015.

80. Kozikowski AP, Onajole OK, Stec J, Dupont C, Viljoen A, Richard M, Chaira T, Lun S, Bishai W, Raj VS, Ordway D, Kremer L. 2017. Targeting mycolic acid transport by indole-2-carboxamides for the treatment of *Mycobacterium abscessus* infections. J Med Chem 60:5876–5888. https://doi.org/10.1021/acs.jmedchem.7b00582.

81. Pandya AN, Prathipati PK, Hegde P, Li W, Graham KF, Mandal S, Drescher KM, Destache CJ, Ordway D, Jackson M, North EJ. 2019. Indole-2-carboxamides are active against *Mycobacterium abscessus* in a mouse model of acute infection. Antimicrob Agents Chemother 63:e02245-18. https://doi.org/10.1128/AAC.02245-18.

82. de Ruyck J, Dupont C, Lamy E, Le Moigne V, Biot C, Guérardel Y, Herrmann J-L, Blaise M, Grassin-Delyle S, Kremer L, Dubar F. 2020. Structure-based design and synthesis of piperidinol-containing molecules as new *Mycobacterium abscessus* inhibitors. ChemistryOpen 9:351–365. https://doi.org/10.1002/open.202000042.

83. Lee S, Jeon BY, Bardarov S, Chen M, Morris SL, Jacobs WR, Jr. 2006. Protection elicited by two glutamine auxotrophs of *Mycobacterium tuberculosis* and *in vivo* growth phenotypes of the four unique glutamine synthetase mutants in a murine model. Infect Immun 74:6491–6495. https://doi.org/10.1128/IAI.00531-06.

84. Tullius MV, Harth G, Horwitz MA. 2003. Glutamine synthetase GlnA1 is essential for growth of *Mycobacterium tuberculosis* in human THP-1 macrophages and guinea pigs. Infect Immun 71:3927–3936. https://doi.org/10.1128/iai.71.7.3927-3936.2003.

85. Turner KH, Wessel AK, Palmer GC, Murray JL, Whiteley M. 2015. Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. Proc Natl Acad Sci U S A 112:4110–4115. https://doi.org/10.1073/pnas.1419677112.

86. Visentin M, Zhao R, Goldman ID. 2012. The antifolates. Hematol Oncol Clin North Am 26:629–648. https://doi.org/10.1016/j.hoc.2012.02.002.

87. Hagner N, Joerger M. 2010. Cancer chemotherapy: targeting folic acid synthesis. Cancer Manag Res 2:293–301. https://doi.org/10.2147/CMR.S10043.

88. Nixon MR, Saionz KW, Koo M-S, Szymonifka MJ, Jung H, Roberts JP, Nandakumar M, Kumar A, Liao R, Rustad T, Sacchettini JC, Rhee KY, Freundlich JS, Sherman DR. 2014. Folate pathway disruption leads to critical disruption of methionine derivatives in *Mycobacterium tuberculosis*. Chem Biol 21:819–830. https://doi.org/10.1016/j.chembiol.2014.04.009.

89. Cheng YS, Sacchettini JC. 2016. Structural insights into *Mycobacterium tuberculosis* Rv2671 protein as a dihydrofolate reductase functional analogue contributing to para-aminosalicylic acid resistance. Biochemistry 55:1107–1119. https://doi.org/10.1021/acs.biochem.5b00993.

90. Zheng J, Rubin EJ, Bifani P, Mathys V, Lim V, Au M, Jang J, Nam J, Dick T, Walker JR, Pethe K, Camacho LR. 2013. *para*-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in *Mycobacterium tuberculosis*. J Biol Chem 288:23447–23456. https://doi.org/10.1074/jbc.M113.475798.

91. Rodionova IA, Schuster BM, Guinn KM, Sorci L, Scott DA, Li X, Kheterpal I, Shoen C, Cynamon M, Locher C, Rubin EJ, Osterman AL. 2014. Metabolic and bactericidal effects of targeted suppression of NadD and NadE enzymes in mycobacteria. mBio 5:e00747-13. https://doi.org/10.1128/mBio.00747-13.

92. Kim J-H, O'Brien KM, Sharma R, Boshoff HIM, Rehren G, Chakraborty S, Wallach JB, Monteleone M, Wilson DJ, Aldrich CC, Barry CE, Rhee KY, Ehrt S, Schnappinger D. 2013. A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. Proc Natl Acad Sci U S A 110:19095–19100. https://doi.org/10.1073/pnas.1315860110.

93. Wang X, Ahn Y-M, Lentscher AG, Lister JS, Brothers RC, Kneen MM, Gerratana B, Boshoff HI, Dowd CS. 2017. Design, synthesis, and evaluation of substituted nicotinamide adenine dinucleotide (NAD$^+$) synthetase inhibitors as potential antitubercular agents. Bioorg Med Chem Lett 27:4426–4430. https://doi.org/10.1016/j.bmcl.2017.08.012.

94. Vollmer W, Joris B, Charlier P, Foster S. 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev 32:259–286. https://doi.org/10.1111/j.1574-6976.2007.00099.x.

95. Beiko RG, Harlow TJ, Ragan MA. 2005. Highways of gene sharing in prokaryotes. Proc Natl Acad Sci U S A 102:14332–14337. https://doi.org/10.1073/pnas.0504068102.

96. Kloesges T, Popa O, Martin W, Dagan T. 2011. Networks of gene sharing among 329 proteobacterial genomes reveal differences in lateral gene transfer frequency at different phylogenetic depths. Mol Biol Evol 28:1057–1074. https://doi.org/10.1093/molbev/msq297.

97. Merianos HJ, Wang J, Moore PB. 2004. The structure of a ribosomal protein S8/spc operon mRNA complex. RNA 10:954–964. https://doi.org/10.1261/rna.7030704.

98. Wower I, Kowaleski MP, Sears LE, Zimmermann RA. 1992. Mutagenesis of ribosomal protein S8 from *Escherichia coli*: defects in regulation of the *spc* operon. J Bacteriol 174:1213–1221. https://doi.org/10.1128/jb.174.4.1213-1221.1992.

99. Bloom-Ackermann Z, Navon S, Gingold H, Towers R, Pilpel Y, Dahan O. 2014. A comprehensive tRNA deletion library unravels the genetic architecture of the tRNA pool. PLoS Genet 10:e1004084. https://doi.org/10.1371/journal.pgen.1004084.

100. Kim YS, Yang C-S, Nguyen LT, Kim JK, Jin HS, Choe JH, Kim SY, Lee H-M, Jung M, Kim J-M, Kim MH, Jo E-K, Jang J-C. 2017. *Mycobacterium abscessus* ESX-3 plays an important role in host inflammatory and pathological responses during infection. Microbes Infect 19:5–17. https://doi.org/10.1016/j.micinf.2016.09.001.

101. Lamichhane G, Zignol M, Blades NJ, Geiman DE, Dougherty A, Grosset J, Broman KW, Bishai WR. 2003. A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 100:7213–7218. https://doi.org/10.1073/pnas.1231432100.

102. Long JE, DeJesus M, Ward D, Baker RE, Ioerger T, Sassetti CM. 2015. Identifying essential genes in *Mycobacterium tuberculosis* by global phenotypic profiling. Methods Mol Biol 1279:79–95. https://doi.org/10.1007/978-1-4939-2398-4_6.

103. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

104. Xu L, Dong Z, Fang L, Luo Y, Wei Z, Guo H, Zhang G, Gu YQ, Coleman-Derr D, Xia Q, Wang Y. 2019. OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species. Nucleic Acids Res 47:W52–W58. https://doi.org/10.1093/nar/gkz333.

105. Naville M, Ghuillot-Gaudeffroy A, Marchais A, Gautheret D. 2011. ARNold: a web tool for the prediction of Rho-independent transcription terminators. RNA Biol 8:11–13. https://doi.org/10.4161/rna.8.1.13346.