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Conditional Depletion of KasA, a Key Enzyme of Mycolic Acid Biosynthesis, Leads to Mycobacterial Cell Lysis

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Inhibition or inactivation of InhA, a fatty acid synthase II (FASII) enzyme, leads to mycobacterial cell lysis. To determine whether inactivation of other enzymes of the mycolic acid-synthesizing FASII complex also leads to lysis, we characterized the essentiality of two β-ketoacyl–acyl carrier protein synthases, KasA and KasB, in *Mycobacterium smegmatis*. Using specialized transduction for allelic exchange, null *kasB* mutants, but not *kasA* mutants, could be generated in *Mycobacterium smegmatis*, suggesting that unlike *kasB*, *kasA* is essential. To confirm the essentiality of *kasA*, and to detail the molecular events that occur following depletion of KasA, we developed CESTET (*c*onditional *e*xpression *s*pecialized *t*ransduction *e*ssentiality *t*est), a genetic tool that combines conditional gene expression and specialized transduction. Using CESTET, we were able to generate conditional null *inhA* and *kasA* mutants. We studied the effects of depletion of KasA in *M. smegmatis* using the former strain as a reference. Depletion of either InhA or KasA led to cell lysis, but with different biochemical and morphological events prior to lysis. While InhA depletion led to the induction of an 80-kDa complex containing both KasA and AcpM, the mycobacterial acyl carrier protein, KasA depletion did not induce the same complex. Depletion of either InhA or KasA led to inhibition of α and epoxy mycolate biosynthesis and to accumulation of α′-mycolates. Furthermore, scanning electron micrographs revealed that KasA depletion resulted in the cell surface having a "crumpled" appearance, in contrast to the blebs observed on InhA depletion. Thus, our studies support the further exploration of KasA as a target for mycobacterial-drug development.

The emergence of multidrug-resistant *Mycobacterium tuberculosis* has been partly responsible for the global spread of tuberculosis (TB) in the past decade (51). There is an urgent need to develop novel drugs that are active against *M. tuberculosis*. Existing antimycobacterial agents act either by inhibiting growth or by causing cell death. The antituberculosis drug isoniazid (INH) inhibits the biosynthesis of mycolic acids, major components of cell wall lipids, resulting in cell death by lysis (43, 49, 50). INH inhibits InhA (1, 21, 32), an enoyl-acyl carrier protein (ACP) reductase which is part of the multienzyme fatty acid synthase II complex (FASII) that synthesizes the meromycolate precursors of mycolic acids. Although the structures of mycolic acids have been well characterized, the genetics and enzymology of mycolic acid biosynthesis have only recently begun to be elucidated (1, 2, 15, 20, 32, 36, 37, 42, 47). Mycobacteria, unlike most bacteria, have two fatty acid synthases. The eukaryote-like FASI, which is a large multidomain polypeptide containing all enzymatic functions required for de novo fatty acid synthesis, produces in a bimodal fashion saturated fatty acids of palmitate ($C_{16:0}$) and tetracosanoate ($C_{24:0}$) or C_{26} (6, 30). FASII elongates FASI end products to form long-chain fatty acids or meromycolates. Acyl chains bound to an ACP are elongated by repetitive cycles of condensation, keto reduction, dehydration, and enoyl reduction catalyzed by a β-ketoacyl-ACP synthase, a β-ketoacyl-ACP reductase, a β-hydroxyacyl-ACP dehydratase, and an enoyl-ACP reductase, respectively. The resultant meromycolate chain is then condensed with a C_{26} fatty acid to form a mycolic acid (31). In *M. tuberculosis*, *M. bovis*, and *M. smegmatis*, genes known to encode FASII enzymes are found in two loci (2, 9, 16). One locus is an operon which includes genes encoding two discrete β-ketoacyl-ACP synthases (*kasA* and *kasB*) and the acyl carrier protein (*acpM*). The ketoreductase (*mabA*) and the enoyl reductase (*inhA*) genes are present in another operon (1, 2). Although open reading frames encoding putative dehydratases have been identified in mycobacterial genomes (42), their roles in FASII have yet to be tested.

Exploiting a mutation in *inhA* that made the InhA protein thermolabile, Vilchèze et al. (47) were able to demonstrate that the thermal inactivation of InhA was sufficient to induce the lysis of *M. smegmatis*. However, it was unclear if lysis was a direct result of inactivation/inhibition of InhA or an indirect result caused by the inactivation of the FASII pathway. In other words, it was not known whether the inactivation of other FASII enzymes would also lead to lysis. Taking a genetic approach to address the above question, we targeted the two β-ketoacyl–ACP synthases KasA and KasB, which catalyze the first step of the reductive cycle. Our aim was to first determine

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TABLE 1. Plasmids, bacterial strains, and phages used in this study

Plasmid, strain, or phage	Description[a]	Reference/source
Plasmids		
pMV306	Kan^r, single-copy-integrating vector; inserts into the phage L5 chromosomal integrating site att_B in many mycobacteria	40
pCR2.1-TOPO	PCR product cloning vector	Invitrogen Life Technologies
pYUB2411	*inhA* cloned downstream of the acetamidase promoter in pMV306	This work
pYUB2412	*kasA* cloned downstream of the acetamidase promoter in pMV306	This work
pJSC347	Vector for cloning allelic-exchange substrates to be used for specialized transduction; contains λ phage *cos* site and Hyg^r marker	33
pYUB2413	Derivative of pJSC347 designed for allelic exchange of *M. smegmatis inhA*	This work
pYUB2414	Derivative of pJSC347 designed for allelic exchange of *M. smegmatis kasA*	This work
pYUB2415	Derivative of pJSC347 designed for allelic exchange of *M. smegmatis kasB*	This work
pYUB2416	*M. smegmatis inhA* cloned into pSD26 (10), an expression vector with the acetamidase promoter	Gift from C. Vilchèze
Bacterial strains		
mc^{2}155	Electroporation-proficient *ept* mutant of *M. smegmatis* strain mc^{2}6	39
mc^{2}155::pYUB411	Kan^r mc^{2}155 derivative containing pYUB2411 integrated into the att_B site	This work
mc^{2}155::pYUB412	Kan^r mc^{2}155 derivative containing pYUB2412 integrated into the att_B site	This work
mc^{2}4751	Chromosomal copy of *inhA* in mc^{2}155::pYUB2411 is replaced by a hygromycin resistance cassette	This work
mc^{2}4752	Chromosomal copy of *kasA* in mc^{2}155::pYUB2411 is replaced by a hygromycin resistance cassette	This work
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from the lytic mycobacteriophage TM4	Gift from J. Kriakov
phAE401	Derivative of phAE159 obtained by cloning pYUB2413 into its unique PacI site	This work
phAE402	Derivative of phAE159 obtained by cloning pYUB2414 into its unique PacI site	This work
phAE403	Derivative of phAE159 obtained by cloning pYUB2415 into its unique PacI site	This work

[a] att_B, phage L5 chromosomal integration site.

whether *kasA* and *kasB* were essential in *M. smegmatis*. By specialized transduction, a highly efficient phage-based allelic-exchange method (3), *kasB* was shown to be nonessential for the in vitro growth of *M. smegmatis* mc^{2}155. This was consistent with studies performed with *Mycobacterium marinum* (15). In contrast, a specialized transducing phage designed to delete *kasA* did not yield any transductants in *M. smegmatis* mc^{2}155, suggesting that *kasA* is essential for growth. To demonstrate the essentiality of *kasA*, we developed a gene essentiality testing method designated CESTET (*c*onditional *e*xpression *s*pecialized *t*ransduction *e*ssentiality *t*est). CESTET extends the utility of the inducible acetamidase promoter (26, 29) by using it in conjunction with specialized transduction. The validity of the method was evaluated using the essential *inhA* gene, and CESTET was then used to demonstrate the essentiality of *kasA* by generating conditional null mutations in *M. smegmatis*. Conditional depletion of KasA established that the effects of loss of KasA were bactericidal.

MATERIALS AND METHODS

Plasmids, phages, and bacterial strains. Plasmids, phages, and bacterial strains used in this study are outlined in Table 1. Strains of *Mycobacterium smegmatis* were grown in tryptic soy broth (TSB; Difco) containing 0.05% Tween 80 (TSBT) or in Sauton medium. *Escherichia coli* strains were cultured in LB broth. Solid media were made by adding 1.5% agar to the above-mentioned broths. The concentrations of antibiotics used were 100 μg/ml for hygromycin and 20 μg/ml for kanamycin with *M. smegmatis* and 150 μg/ml for hygromycin and 40 μg/ml for kanamycin with *E. coli*.

Construction of merodiploid strains. The single-copy-integrating construct pYUB2411 was constructed by ligating a 3.5-kb XbaI-ClaI fragment from plasmid pYUB2416 (containing the *M. smegmatis inhA* gene cloned in frame, downstream of the inducible *M. smegmatis* acetamidase promoter) into XbaI-ClaI-digested pMV306. The *M. tuberculosis kasA* open reading frame was PCR amplified from cosmid DNA using the primers KASA-L (GCGAGATCTGTG ACCAGGCCTTCCACTG) and KASA-R (GCATTCGAATCAGTAACGCCC GAATGC) and cloned into pCR2.1-TOPO (Invitrogen Life Technologies). The plasmid pYUB2412 was constructed by simultaneous ligation of a 1.8-kb XbaI-BamHI acetamidase promoter fragment and a BglII-HindIII *kasA* fragment (pCR2.1-TOPO clone) into XbaI-HindIII-digested pMV306. Merodiploid strains mc^{2}155::pYUB2411 and mc^{2}155::pYUB2412 were obtained by electroporating mc^{2}155 with pYUB2411 and pYUB2412, respectively, and selecting for kanamycin-resistant colonies.

Construction of deletion mutants. Approximately 1-kb sequences flanking the left and right portions of the *inhA* gene were PCR amplified from *M. smegmatis* genomic DNA using the primer pairs MSInh1 (GCAGATCTGATATCACGC GAGCGTCGGCTGGC) and MSInh2 (TCTAGACTCGAGGCGGTCGAAAC CGGTCAG) and MSInh3 (GCTCTAGAGCGATGAGCGCAATCGTG) and MSInh4 (GCCCATGGATATCGTCGGCGACGAAGCCGAG), respectively. Following cloning into pCR2.1-TOPO and sequencing, the cloned PCR fragments were excised using the primer-introduced restriction sites and cloned into the allelic-exchange plasmid vector pJSC347 (Table 1). The resultant plasmid, pYUB2413, was then packaged into the temperature-sensitive phage phAE159 (J. Kriakov and W. R. Jacobs Jr., unpublished results) as described earlier (3) to yield the knockout phage phAE401. Knockout plasmids and phages for *M. smegmatis kasA* (pYUB2414 and phAE402) and *kasB* (pYUB2415 and phAE403) were generated in a similar fashion. The primers pairs used for amplification of ~1 kb of sequences flanking *kasA* were KASA1 (GTCACTAG TAGATCGTCGCC) and KASA2 (CTGTTCGAAACGCGGATACCGCTCTC GCCG), and KASA3 (GACTCTAGACAGGTGAGCCTCGGTACG) and KASA4 (TGCGGTACCGTGCCGGTCGCATGCGCG). The primers pairs used for amplification of ~1 kb of sequences flanking *kasB* were KASB1 (GATACT

AGTCAAGGTGTCGCCGCTCGC) and KASB2 (GCGTTCGAACTCTGGCC GTCGAGCAGC) and KASB3 (GTATCTAGACCACGACAGGGCGATTAC) and KASB4 (GCAGGTACCGGTTGTTGGCGATGACAC). Specialized transduction was performed as described previously (3), except that for essential gene testing, in which the host strains were the respective merodiploids and half the transduction mix was spread on TSB agar plates containing kanamycin, hygromycin, and 0.2% acetamide, while the other half was spread on plates lacking acetamide.

Conditional depletion of InhA or KasA. Strains to be tested were grown in TSBT containing 0.2% acetamide to an A_{600} of 0.5. The cells were washed twice in TSBT to remove traces of acetamide and resuspended to the original volume in TSBT. This cell suspension was used as a 20% inoculum in TSBT and grown for 12 h to deplete intracellular InhA or KasA. The depleted culture was then used to inoculate TSBT with or without 0.2% acetamide (5% inoculum). This inoculation of the depleted culture corresponds to the 0-h time point for all experiments designed to test cell density and viability and morphological changes (see below). The same procedure was followed for a culture grown in Sauton medium to test the biochemical effects of depletion.

Determination of viable-cell counts. For determining numbers of CFU, aliquots of cultures were serially diluted in TSBT (10-fold dilutions). The appropriate dilutions were then spread on TSB agar containing 0.2% acetamide, and the plates were incubated at 37°C for 72 h before CFU were counted.

Western blotting. Following inoculation from a 12-h-depleted culture (see above), bacterial cells were grown in Sauton medium in the absence or presence of acetamide and harvested at different time points. The cell pellet was resuspended in 0.8 ml of phosphate-buffered saline (20 mM K_2HPO_4, pH 7.5, 0.15 M NaCl) and disrupted for 10 min using a model 450 Branson sonifier. Protein concentrations were determined using the BCA protein assay reagent kit (Pierce), and equal amounts (20 μg) were then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Proteins were then transferred onto a membrane, which was subsequently saturated with 2% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20. Membranes were then probed with either rat anti-KasA antibodies (22) or rabbit anti-InhA antibodies (21) and incubated with the appropriate alkaline phosphatase-conjugated antibodies.

In vivo effects of InhA or KasA depletion on mycolic acid synthesis. Using the depleted culture grown for 12 h in Sauton medium without acetamide as the inoculum (see above), cultures were grown in Sauton medium in the presence or absence of acetamide. Aliquots were taken after 7 and 24 h and labeled with 1 μCi/ml of [1-^{14}C]acetate (50 to 62 mCi/mmol; Amersham) for 3 h. Cells were then harvested by centrifugation, washed with water, and stored at −20°C. Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were obtained by treatment of the radiolabeled cell pellets with aqueous tetrabutyl ammonium hydroxide, esterification with methyl iodide, and extraction with dichloromethane as described previously (19). Equal counts (50,000) were spotted on a thin-layer chromatography (TLC) plate, which was developed in petroleum ether-acetone (95%-5%, vol/vol) and exposed overnight to X-ray films. Two-dimensional argentation TLC analysis of the 24-h samples was done as described earlier (22).

SEM. Using the depleted culture grown for 12 h in TSBT without acetamide as the inoculum (see above), samples for scanning electron microscopy (SEM) were grown in TSBT (with or without acetamide) for 24 h. One milliliter of culture was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, applied to poly-L-lysine-coated coverslips, dehydrated through a graded series of ethanol, critical point dried using liquid carbon dioxide in a model 790 Tousimis Samdri (Rockville, Md.) critical point drier, and then sputter coated with gold-palladium in a Denton (Cherry Hill, N.J.) vacuum desk-1 sputter coater. The samples were examined in a JEOL (Peabody, Mass.) JSM6400 scanning electron microscope using an accelerating voltage of 10 kV.

RESULTS

***The kasA*-encoded β-ketoacyl–ACP synthase is essential in *M. smegmatis*.** Specialized transduction (3) of *M. smegmatis* mc^{2}155 with a temperature-sensitive phage containing an allelic-exchange substrate designed to replace *kasA* with a hygromycin resistance cassette (*hyg*) failed to yield any hygromycin-resistant (Hyg^r) transductants (data not shown). This negative result suggested that KasA is essential for the normal growth of *M. smegmatis*. In contrast, the other β-ketoacyl–ACP synthase, KasB, is dispensable in *M. smegmatis* because null mutants could be generated following specialized transduction with the *kasB* phage phAE403 (data not shown). This result is consistent with the reported nonessentiality on *kasB* in *Mycobacterium marinum* (15).

The failure to obtain *kasA* null mutants did not prove that *kasA* is essential, nor did it allow us to study the fate of mycobacterial cells depleted of KasA. To achieve these objectives, we developed a gene essentiality testing method that adapted the inducible acetamidase promoter (26, 29) for use in conjunction with specialized transduction. The gene essentiality testing system was called CESTET to distinguish it from other methods that make use of the acetamidase promoter (12, 17). CESTET involves three steps: (i) the construction of a merodiploid strain containing a second copy of a putative essential gene fused transcriptionally to the acetamidase promoter, (ii) the generation of a specialized transducing phage containing an allelic-exchange substrate designed to replace the target gene with *hyg*, and (iii) transduction of the merodiploid strain prior to growth in the presence or absence of acetamide (Fig. 1).

We first validated CESTET using *inhA*, a mycolic acid biosynthesis gene known to be essential (47). When transduced with phAE401, Hyg^r colonies of the *inhA* merodiploid strain (mc^{2}155::pYUB2411) could be obtained only if the transduction mix was plated on selective TSB agar containing 0.2% acetamide (Fig. 2). Between 28 and 63 Hyg^r colonies were obtained on acetamide-containing plates from four independent transductions (average number of Hyg^r colonies, 41). Southern blot analysis of genomic DNA from four independent Hyg^r transductants selected from one transduction experiment revealed replacement of the wild-type chromosomal copy of *inhA* by *hyg* (Fig. 3a). [^{32}P]dCTP-labeled probes derived from the ~1-kb flanking sequences hybridized with 9.3-kb and 11.7-kb BspHI bands in the parental merodiploid strain, while as expected, all four Hyg^r transductants produced a single BspHI band of 22.2 kb (Fig. 3a). One of the Hyg^r transductants designated mc^{2}4751 was selected for further work. Thus, the survival of cells that had undergone allelic exchange to delete *inhA* was dependent on the acetamidase promoter-driven expression of the extra copy of *inhA*, first, confirming that *inhA* was an essential gene and second, validating the essentiality test.

When the *kasA* knockout phage phAE402 was transduced into the *kasA* merodiploid strain mc^{2}155::pYUB2412, between 31 and 50 Hyg^r colonies (average number of Hyg^r colonies, 39) were obtained on TSB agar plates containing acetamide from four different transductions, but none were obtained on TSB agar plates without acetamide (Fig. 2, bottom panels). Southern blot analysis confirmed replacement of the chromosomal copy of *kasA* by *hyg* (Fig. 3b). [^{32}P]dCTP-labeled probes derived from ~1-kb flanking sequences hybridized with a 6.7-kb PstI band in the parental merodiploid strain and with two PstI bands of sizes 2.3 kb and 5.3 kb in the three independent Hyg^r transductants tested (Fig. 3b). One of the conditional *kasA* deletion mutants, designated mc^{2}4752, was selected for further work. No Hyg^r colonies were obtained when phAE402 was transduced into an mc^{2}155 strain containing *kasB* cloned on an autonomously replicating plasmid, indicating that extra copies of *kasB* could not compensate for the loss of *kasA* (data not shown). These results show that *kasA* is an essential gene in *M.*

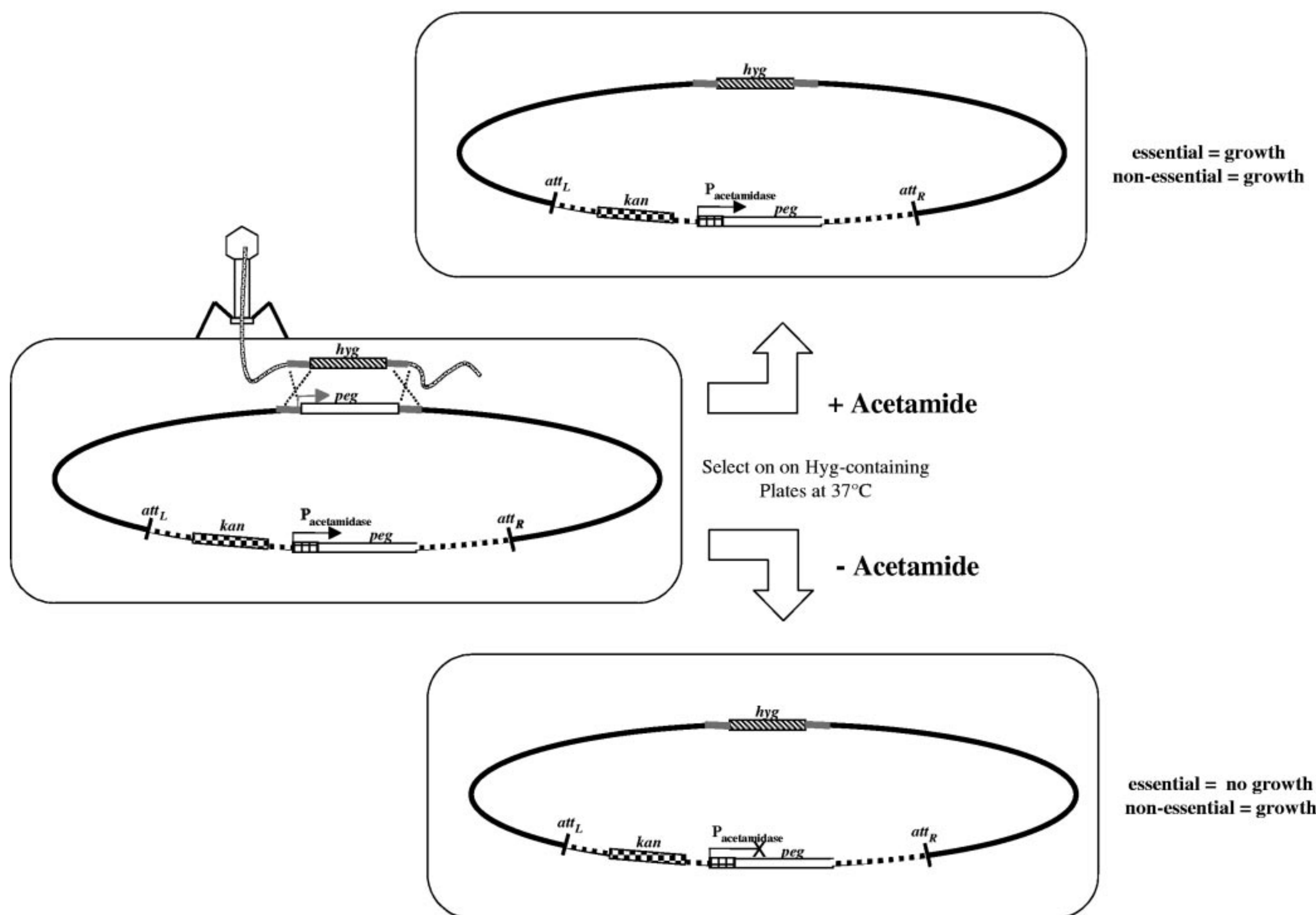


FIG. 1. Schematic representation of CESTET. Following the injection of recombinant phage DNA into a merodiploid strain, allelic exchange mediated by recombination of homologous sequences (regions in gray) results in deletion of the natural copy of a putative essential gene (*peg*). The transduction mix is split into two equal parts, which are plated on hygromycin-containing medium with or without acetamide. If the gene is essential, transductants are expected to grow only on plates containing acetamide. att_L and att_R, left and right junctions of the phage L5 attachment and chromosomal integration site; *kan*, kanamycin resistance cassette; *hyg*, hygromycin resistance cassette; Hyg, hygromycin; $P_{acetamidase}$, inducible acetamidase promoter. The interrupted arc represents the integrative vector, the solid arc represents the mc^{2}155 chromosome, and the dotted strand represents phage DNA.

smegmatis and that KasA and KasB have nonredundant roles in mycolic acid biosynthesis.

Conditional depletion of KasA is a bactericidal event that leads to the lysis of *M. smegmatis*. Previous studies demonstrated that the thermal inactivation of mutant InhA protein led to cessation of mycolic acid synthesis, followed by cell lysis (47). While thermal inactivation of a thermolabile enzyme would be expected to inactivate every molecule of the mutated peptide at the nonpermissive temperature, observing the effects of conditional depletion of a protein would require turnover and a dilution of existing functional protein. To achieve this, the conditional *inhA* mutant mc^{2}4751 was grown to mid-log phase (12 h) in the absence of acetamide, prior to inoculation in medium with or without acetamide. Optical density (OD) measurements revealed normal growth kinetics for mc^{2}4751 cells grown in acetamide-containing medium. However, mc^{2}4751 cells cultured in the absence of acetamide showed a decrease in OD after 12 h of growth. After 18 h there was clearing in the growth medium accompanied by an accumulation of debris indicating cell lysis (Fig. 4 inset). This decrease in OD correlated with a decrease in intracellular levels of InhA (Fig. 5a) as well as a decrease in the number of CFU (Fig. 4a). Thus, InhA depletion had the same effect as INH treatment or thermal inactivation on a thermolabile mutant InhA.

We then analyzed the growth kinetics of the conditional *kasA* mutant mc^{2}4752. Growth in the absence of acetamide resulted in the loss of KasA (Fig. 5b), which correlated with a drop in the OD and a decrease in numbers of CFU (Fig. 4b). At 18 h, the culture showed complete lysis (Fig. 4b, insets). This result established that depletion of KasA was sufficient for the lysis of *M. smegmatis*.

Conditional depletion of InhA or KasA leads to different biochemical and morphological phenotypes prior to lysis. An understanding of the biochemical and morphological events that precede mycobacterial cell lysis might lead to ways to augment lytic pathways. Phenotypes of lysing mycobacterial cells have been described in detail for mycobacteria treated with INH (1, 21, 43, 44, 47). Previous studies have shown that INH treatment correlates with (i) inhibition of mycolic acid

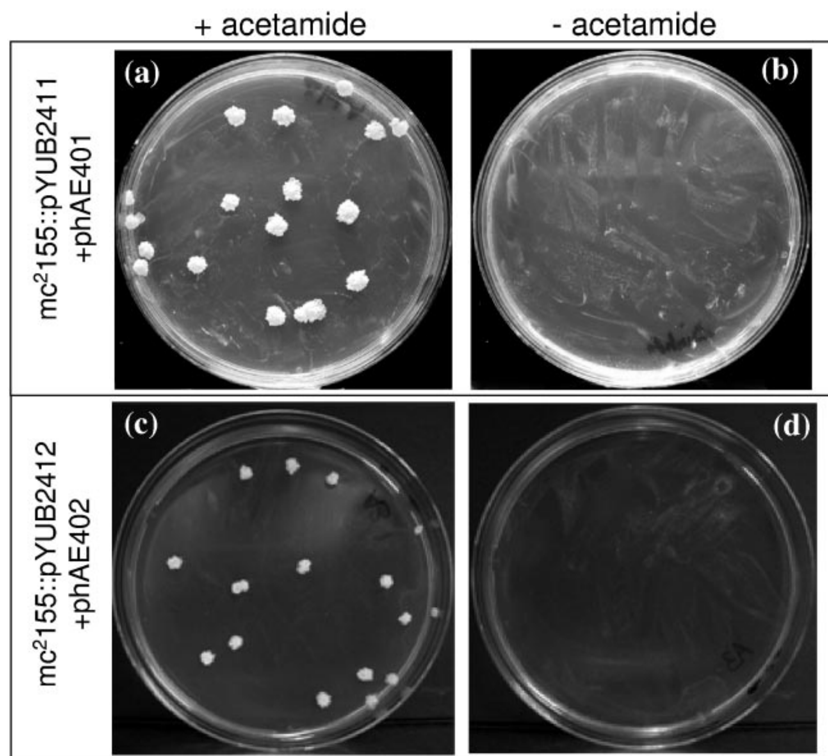


FIG. 2. CESTET to demonstrate the essentiality of *M. smegmatis inhA* (top panels) or *kasA* (bottom panels). Transduction mixes (10^9 CFU mixed with 10^{10} PFU) are indicated on the left. Each mix was split equally and plated out on hygromycin-containing TSB agar with (left panels) or without (right panels) acetamide. Each plate represents one-fourth of the transduction mix. Hyg^r colonies can be seen only on acetamide-containing plates.

biosynthesis, (ii) accumulation of an 80-kDa complex containing both KasA and AcpM, and (iii) formation of blebs on the cell surface, followed by cell lysis (21, 28, 41, 43, 44). Although depletion of KasA also led to cell lysis, it was unclear whether morphological and biochemical phenotypes prior to lysis were similar to those described for INH treatment. We thus compared the effects of KasA depletion to those of InhA depletion by monitoring the above correlates of INH treatment.

An 80-kDa KasA-containing complex was induced when *M. smegmatis* was grown in the presence of InhA-inhibitory drugs, including INH (21), but not in the presence of thiolactomycin (TLM), a known inhibitor of KasA (19, 38). Moreover, several InhA(Ts) mutant strains of *M. smegmatis* constitutively expressed the KasA-containing complex, suggesting that a defect of InhA activity induces this complex (21). Both $mc^2$4751 and $mc^2$4752 were tested for induction of the KasA-containing complex following growth in medium lacking acetamide. Western blots using anti-*M. tuberculosis* KasA antibodies showed that a basal level of the complex was preexistent in both strains (Fig. 6, lanes with acetamide). This finding was not unusual, as the KasA-containing complex has been detected before in untreated mycobacteria, suggesting a physiological role (21). The levels of the KasA-containing complex increased in $mc^2$4751 within 6 h of growth in medium without acetamide (Fig. 6a) but remained steady in cultures grown in the presence of acetamide. In contrast, there was no induction of the KasA-containing complex in $mc^2$4752 cells grown in the absence of acetamide (Fig. 6b). Induction of the 80-kDa complex in the conditional *kasA* mutant would be expected to manifest in a fashion where the decrease in levels of free KasA would be faster than the decrease in levels of the KasA-containing complex. This was not the case, as the Western blot of $mc^2$4752 cells cultured in medium without acetamide showed a proportional decrease in the levels of free KasA and the 80-kDa complex (Fig. 6b). The anti-KasA antibody also cross-reacted with the second ketoacyl-ACP synthase, KasB, a protein highly related to KasA (19, 36) and present in the crude lysates from $mc^2$4751 and $mc^2$4752. Although *kasA* and *kasB* are adjacent genes from the same operon, this result indicates that deletion of *kasA* did not have a polar effect on *kasB* expression. Moreover, Western blots using anti-*M. tuberculosis* InhA antibodies demonstrated that the loss of KasA does not alter InhA expression (Fig. 6b) and, conversely, that depletion of InhA does not affect the expression level of free KasA (Fig. 6a).

The levels of mycolic acid biosynthesis were monitored at different time intervals with $mc^2$4751 and $mc^2$4752 following growth in medium with or without acetamide. Cultures were pulsed with [^{14}C]acetate at different time intervals after inoculation in medium, and MAMEs were extracted and analyzed by TLC. Both mutant strains showed a strong decrease in levels of α and epoxy mycolic acids (Fig. 7) when grown in medium lacking acetamide. In contrast, there was an accumulation of α'-mycolates in both $mc^2$4751 and $mc^2$4752. This was more distinct in the *kasA* mutant, presumably because of the faster lysis of the *kasA* mutant. Prolonged treatment of *M. smegmatis* $mc^2$155 with INH showed that the drug had a similar effect

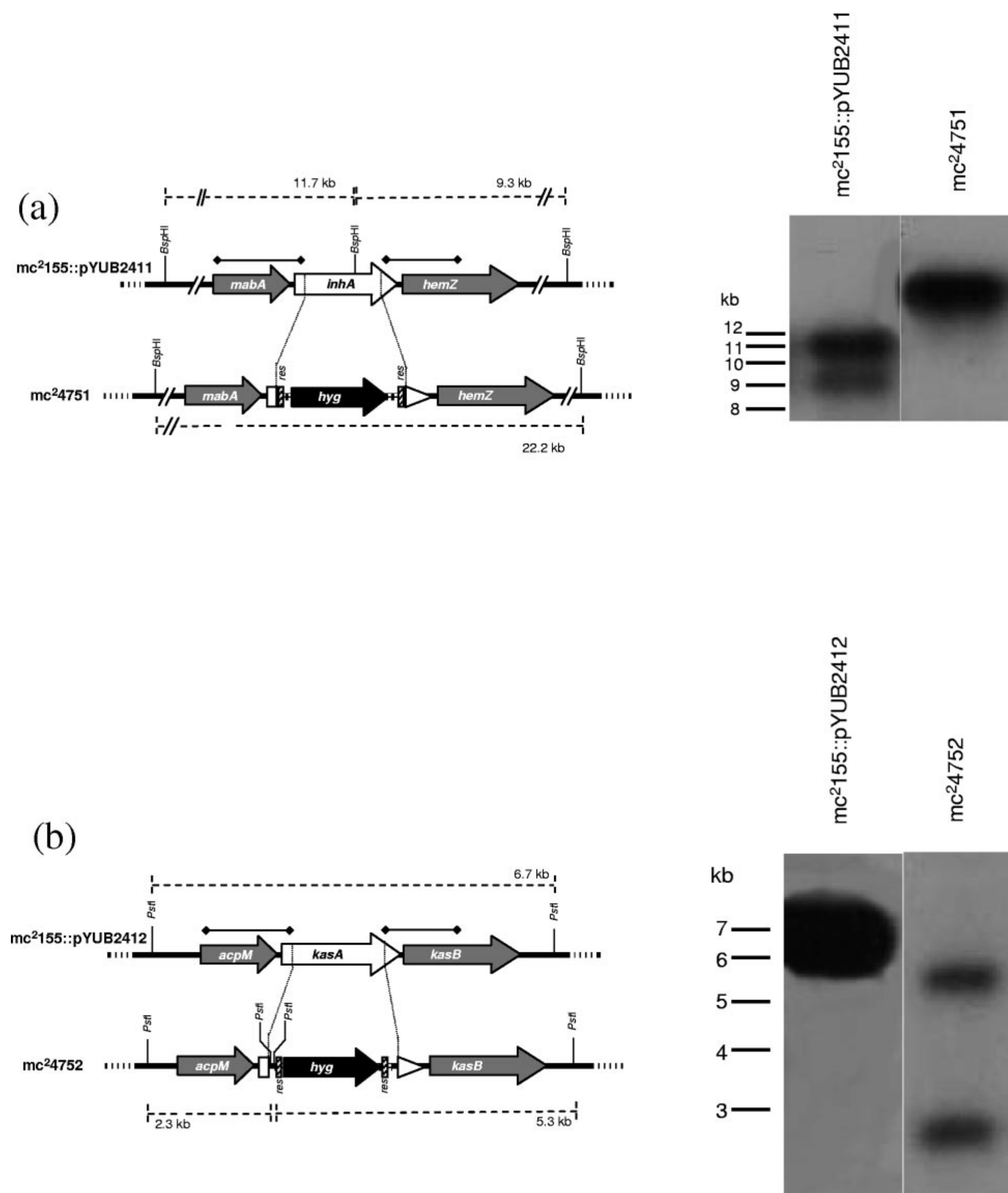


FIG. 3. Southern blots confirming the deletion of *inhA* (a) and *kasA* (b) in mc²155::pYUB2411 and mc²155::pYUB2412, respectively. The adjoining maps show the expected band sizes, and the regions used as probes are indicated as thick lines with square ends. The probes used for each blot were the same (~1 kb) upstream and downstream flanking sequences that were used to construct the knockout plasmids. Only one representative band pattern is shown for each mutant strain. For the *inhA* region, the probes were expected to hybridize with 9.3-kb and 11.7-kb BspHI bands in the parental merodiploid strain and a single BspHI band of 22.2 kb in the *inhA* mutant mc²4751. For the *kasA* region, probes were expected to hybridize with a 6.7-kb PstI band in the parental merodiploid strain and with two PstI bands of sizes 2.3 kb and 5.3 kb in the *kasA* mutant mc²4752. *res*, γδ resolvase site; *hyg*, hygromycin resistance gene.

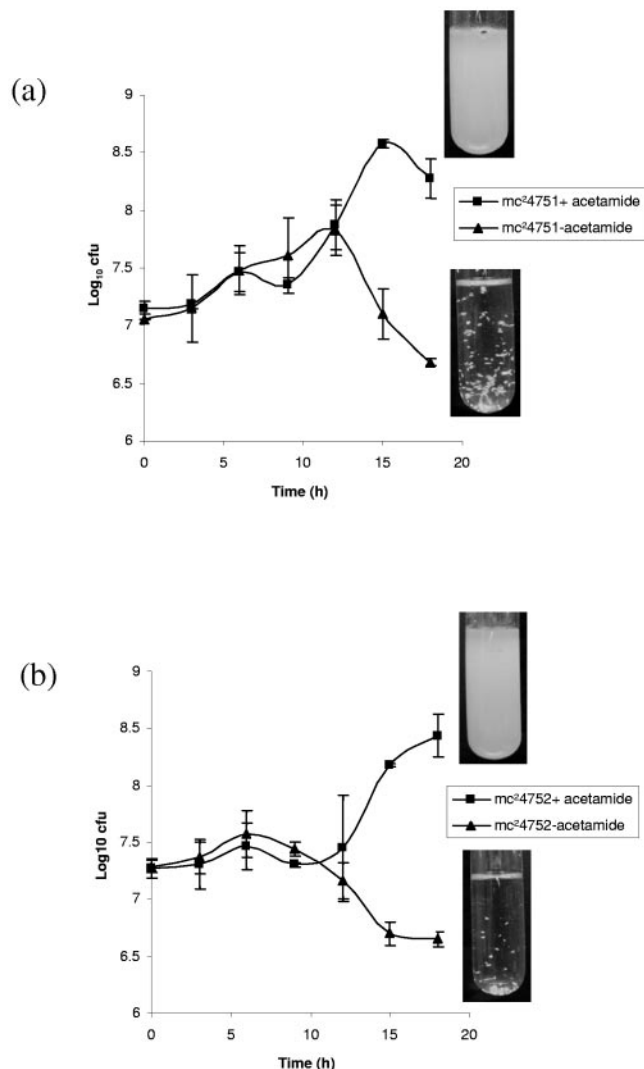
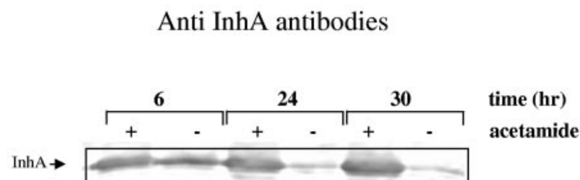


FIG. 4. Plot of numbers of CFU obtained from cultures of mc^{2}4751 (a) and mc^{2}4752 (b) grown in TSBT with or without acetamide, taken at different time points. Log$_{10}$ CFU values are calculated from different dilutions from a single experiment. The insets placed next to the 18-h data points show pictures of the cultures after 18 h of growth in TSBT with or without acetamide. Repetition of the experiment gave similar results (data not shown).

(a)



(b)

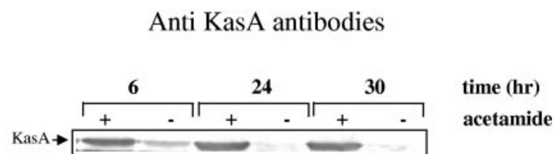


FIG. 5. Western blots of total crude lysates from mc^{2}4751 (a) and mc^{2}4752 (b) grown in the absence or presence of acetamide to detect intracellular levels of InhA (a) or KasA (b) using rabbit anti-InhA or rat anti-KasA antibodies, respectively.

(data not shown). Similarly, treatment of *M. smegmatis* with TLM, a known KasA inhibitor (19, 38), was also accompanied by an accumulation of α'-mycolates (38). Overall, these results indicate that the observed accumulation of α'-mycolates seems to be a general outcome of the loss of FASII activity in *M. smegmatis*. Further analysis of the mycolates by two-dimensional argentation TLC to detect changes in the degrees of unsaturation did not reveal any major differences (data not shown).

Previous studies demonstrated that InhA inactivation, like INH treatment, led to the generation of blebs on the surfaces of bacteria (47). SEMs revealed that conditional depletion of InhA led to extensive bleb formation (Fig. 8b, c, and d). Approximately 77% of the bacteria observed in a total of 20 fields had blebs. In contrast, conditional depletion of KasA in mc^{2}4752 resulted in the cells having an irregular surface with a "crumpled" appearance (Fig. 8f, g, and h). Approximately 84% of the bacteria observed in a total of 20 fields had a "crumpled" appearance. Cells from 24-h cultures of both mutants grown in the presence of acetamide had a normal appearance (Fig. 8a and e). These results indicate that depletion of InhA or KasA resulted in very different morphological changes in the bacteria prior to lysis.

DISCUSSION

In this study, we have demonstrated the essentiality of the FASII component KasA. Furthermore, this is the first description of the effects of depletion of KasA in a mycobacterial cell. We developed a conditional essentiality testing method to demonstrate the essentiality of *inhA* and *kasA* in *M. smegmatis* and then followed the fates of the two mutants by culturing them in medium without acetamide, which resulted in the depletion of InhA or KasA.

While the depletion of either InhA or KasA led to the lysis of mycobacterial cells, the patterns of lysis were profoundly different for the two strains. Growth of wild-type *M. smegmatis* in the presence of INH induces the formation of an 80-kDa KasA-containing complex and causes the appearance of blebs on the cell surface, which leads to cell lysis (21, 47). Similar features were observed when the conditional *inhA* mutant mc^{2}4751 was grown in the absence of acetamide, indicating

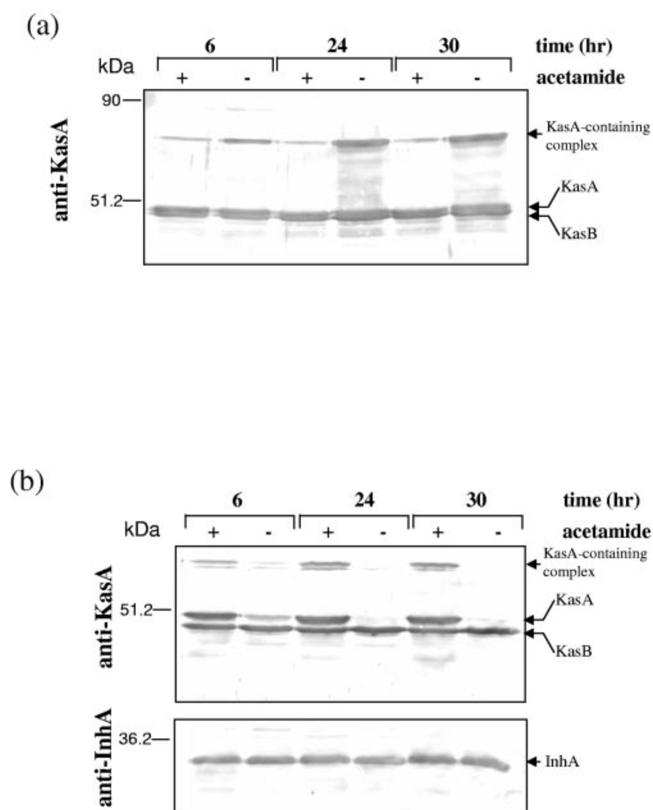


FIG. 6. Western blots of total crude lysates from mc^{2}4751 (a) and mc^{2}4752 (b) to detect the formation of the KasA-containing 80-kDa complex. Detection of KasA and InhA in total protein extracts was performed using rat anti-KasA and rabbit anti-InhA antibodies, whose masses are indicated on the left side of each panel.

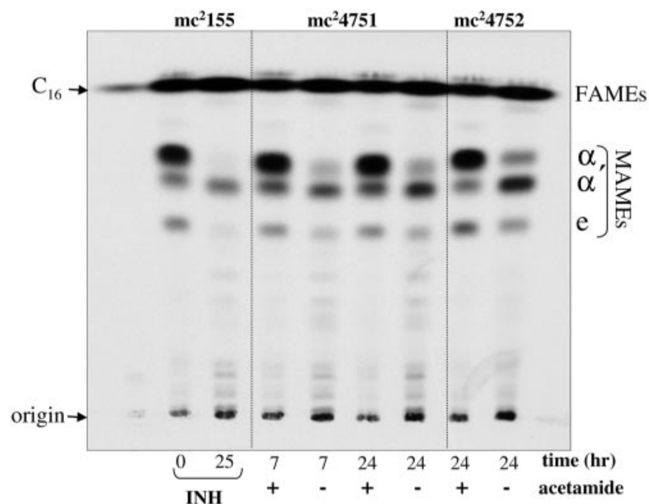


FIG. 7. Thin-layer chromatography of ^{14}C-labeled methyl esters of mycolic acids extracted following depletion of InhA or KasA. Culture aliquots of mc^{2}4751 and mc^{2}4752 were labeled by the addition of [1-^{14}C] acetate (1 μCi/ml) at various time points during growth in the absence or presence of acetamide at 37°C. The control strain, mc^{2}155, was left untreated or treated with 25 μg/ml INH for 3 h. MAMEs and FAMEs were extracted, and equal counts (50,000) were loaded on the TLC plate. Separation was performed as described in Materials and Methods. α, α′, and e correspond to α-mycolates, α′-mycolates, and epoxy mycolates, respectively.

that InhA depletion mimicked InhA inhibition. In contrast, conditional depletion of KasA in mc^{2}4752 led to different phenotypes: there was no induction of the KasA-containing complex and bacterial cells had a crumpled surface appearance prior to lysis. However, in terms of the effects of InhA and KasA depletion on mycolic acid synthesis, the phenotypes appeared comparable. Both strains showed a decrease in the levels of α- and epoxy mycolates, with a concomitant accumulation of α′-mycolates, a feature that has been reported to occur in TLM-treated cells (38).

Our studies also demonstrated that while KasA is essential, the second β-ketoacyl-ACP synthase, KasB, is not an essential component of the FASII machinery. A null *kasB* mutant was generated from a wild-type *M. smegmatis* strain (mc^{2}155), suggesting that KasB is not required for normal growth in vitro. This is consistent with the recent observation that a transposon insertion in *kasB* was not lethal in *Mycobacterium marinum* (15). In agreement with Gao et al. (15), we found that multiple copies of KasB could not compensate for the loss of KasA in *M. smegmatis*, demonstrating that although the two ketosynthases are highly homologous, they do not share redundant functions.

Bacterial cell lysis may not be a direct effect of cessation of mycolic acid biosynthesis. Liu and Nikaido (24) used chemical mutagenesis to isolate a temperature-sensitive mutant of *M. smegmatis* that was unable to synthesize complete mycolic acids but was still viable at 30°C. The mutant strain failed to synthesize full-length mycolates but accumulated incomplete meromycolate chains with a β-hydroxyl group, leading to speculation that the mutation was in the yet-unidentified dehydratase-encoding gene. In addition, this mutant had a cell wall with an abnormal composition and ultrastructure (24, 48). Thus, the lysis observed with the conditional *inhA* and *kasA* mutants may not necessarily be a direct consequence of cell wall damage. Rather, depletion of InhA or KasA may be responsible for changes in cellular metabolic processes, which in turn lead to the induction of specific genes causing bacterial cell death. Indeed, this is the case in *Streptococcus pneumoniae*, in which specific autolysins are induced following penicillin treatment (25, 46). Moreover, the different cell morphologies prior to lysis suggest that the two mutants may have activated different cell death-inducing pathways. In this regard, it has been shown using the DNA microarray technology that although numerous genes were commonly regulated in *M. tuberculosis* treated with INH or TLM (inhibiting InhA and KasA, respectively), there were differences that distinguish the modes of action of these two drugs (5). Therefore, the signature gene expression profiles that discriminate between INH- and TLM-treated mycobacteria may help to identify cell death-inducing genes that are specifically activated following InhA or KasA depletion. A better understanding of these events leading to cell death should allow for the design of compounds that would augment the mycobacterial-killing process. Such compounds could also act synergistically with existing antimicrobials.

To justify high-throughput screening of combinatorial libraries for antimicrobials, it is imperative to characterize an essential gene product as a putative drug target. Furthermore,

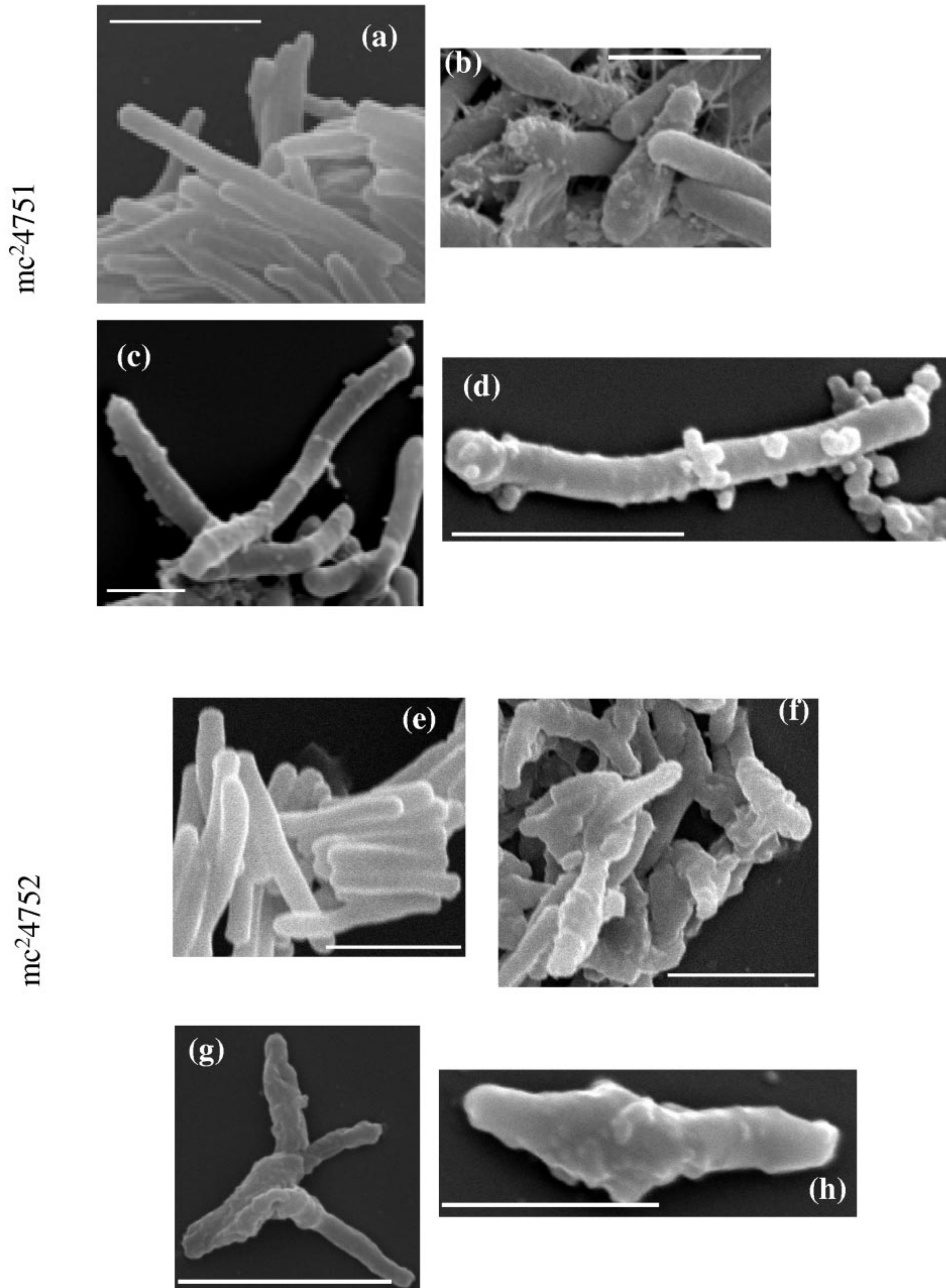


FIG. 8. Scanning electron micrographs of mc^{2}4751 (a, b, c, and d) and mc^{2}4752 (e, f, g, and h). (a and e) Cultures grown for 24 h in the presence of acetamide; (b, c, d, f, g, and h) cultures grown for 24 h in the absence of acetamide. All scale bars represent 0.5 μm.

screening of natural products against a specific target would also require target validation. The demonstration in our studies that the depletion of KasA leads to mycobacterial cell lysis reiterates the importance of TLM and its analogues for future antitubercular therapy (11, 19) and highlights the potential of KasA as an attractive target to be explored for the design of novel drugs.

An additional benefit from our studies was the validation of a gene essentiality testing system that could be used for studying other mycobacterial genes. While transposon mutant screens have identified numerous essential genes (23, 34, 35), they do not provide rigorous proof of the essentiality of individual genes. Data obtained from transposon mutagenesis experiments also do not help distinguish between bacteriostatic and bactericidal effects of gene disruption. Conditional depletion of an essential gene product using CESTET overcomes these limitations. Gomez and Bishai (17) used the chemically inducible acetamidase promoter to establish that WhmD is essential for *M. smegmatis* and leads to filamentous growth upon its conditional depletion. A similar approach was used to follow the fate of cells depleted of FtsZ (12). However, the methods used involved generation of a strain with a single crossover, followed by screening of the second crossover event. We have improved on the utility of the acetamidase promoter to test gene essentiality by combining it with specialized transduction to demonstrate essentiality in a one-step plate assay. Using the *inhA* or *kasA* knockout phages, we consistently obtained scores of independent transductants on plates with acetamide, indicating that the method was reliable for testing gene essentiality. There has been one report to date which describes the inducibility of the *M. smegmatis* acetamidase promoter in *M. tuberculosis* and *Mycobacterium bovis* BCG (27), so it is likely that the essentiality test can also be adapted for use in these slow-growing mycobacteria. Recently, the tetracycline repressor (TetR) and operator (*tetO*)-based system has been used for generating conditional knockouts of essential genes in *M. smegmatis* and for conditional, in vivo expression in *M. tuberculosis* (7, 13). Adapting CESTET to use the TetR-*tetO* system could potentially extend the system for use with slow growers. An added advantage is that gene knockouts are generated using a temperature-sensitive shuttle phasmid (3, 4, 8, 18) derived from the lytic phage TM4 (45), which has a wide host range within mycobacterial species (14), allowing the method to be easily adapted to other mycobacteria. In addition, in slow-growing mycobacteria, illegitimate recombination events commonly encountered with electroporation-based DNA delivery are rarely observed with specialized transduction (3). Our studies also demonstrate that CESTET can be used to validate drug targets, as conditional depletion of a putative target of an antimicrobial drug would be expected to cause phenotypes similar to those seen on treatment of wild-type bacteria with the drug. Moreover, the robustness of specialized transduction makes this system amenable to a high-throughput, whole-genome approach.

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