trometry confirmed that there was greater than 95% incorporation of selenomethionine at the three positions. Difference Patterson maps revealed only two selenium sites, later determined to be at Met^{205} and Met^{255}. A difference Fourier using multiple isomorphous replacement phases failed to reveal the third site, which should have been at Met^{269}. Subsequent determination of the structure showed that there is poor electron density for amino acids 269 to 272, which is in part of the extended loop.
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inhA, a Gene Encoding a Target for Isoniazid and Ethionamide in *Mycobacterium tuberculosis*

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Isoniazid (isonicotinic acid hydrazide, INH) is one of the most widely used antituberculosis drugs, yet its precise target of action on *Mycobacterium tuberculosis* is unknown. A missense mutation within the mycobacterial *inhA* gene was shown to confer resistance to both INH and ethionamide (ETH) in *M. smegmatis* and in *M. bovis*. The wild-type *inhA* gene also conferred INH and ETH resistance when transferred on a multicopy plasmid vector to *M. smegmatis* and *M. bovis* BCG. The InhA protein shows significant sequence conservation with the *Escherichia coli* enzyme EnvM, and cell-free assays indicate that it may be involved in mycolic acid biosynthesis. These results suggest that InhA is likely a primary target of action for INH and ETH.

Despite the availability of effective chemotherapies, tuberculosis is responsible for one in four avoidable adult deaths in developing countries (*1*). Infection with drug-sensitive strains of *M. tuberculosis* can be effectively cured with a combination of INH, rifampicin, and pyrazinamide (*2*). However, the particular susceptibility and increased mortality of the disease among individuals infected with human immunodeficiency virus (HIV) pose a serious threat to tuberculosis control programs (*3*). Moreover, the emergence of multidrug-resistant strains of *M. tuberculosis* (MDR-TB) has resulted in fatal outbreaks in many countries, including the United States (*4*). Strains of MDR-TB, some of which are resistant to as many as seven drugs, are deadly to both HIV^- and HIV^+ individuals (*5*).

INH was first reported to be an effective antituberculosis drug in 1952, displaying particular potency against *M. tuberculosis* and *M. bovis* (*6*). Mutants resistant to INH have emerged since then (*7*), and today such mutants account for as many as 26% of the clinical *M. tuberculosis* isolates in certain U.S. cities (*5*). Some INH-resistant strains are associated with a loss of catalase activity (*8*), and deletions of the catalase-peroxidase gene (*katG*) correlate with INH resistance in certain *M. tuberculosis* isolates (*9*). Furthermore, transfer of the wild-type

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(wt) M. *tuberculosis katG* gene on a multicopy plasmid to INH-resistant M. *smegmatis* and M. *tuberculosis* strains confers INH sensitivity (9), thus confirming previous studies (*10*) suggesting that the catalase-peroxidase activity is required for INH sensitivity. However, only 10 to 25% of INH-resistant isolates from New York or San Francisco appear to be catalase-negative, indicating that INH resistance can be due to other factors (*11*).

Drug resistance can be caused by many mechanisms, including mutations in the drug target that reduce the binding of the drug (*12*) or mutations that lead to increased production of the target (*13*). The mechanism by which INH inhibits M. *tuberculosis* and its precise target of action are unknown. Biochemical evidence has suggested that both INH and ethionamide (ETH, a structural analog of INH and a second-line antituberculosis drug) block mycolic acid biosynthesis in M. *tuberculosis* and other mycobacteria (*14, 15*) at the minimal inhibitory concentration of drug producing >99% reduction of bacterial count (MIC). INH has been found to inhibit mycolic acid biosynthesis in cell-free extracts of mycobacteria (*16*), but the target protein has not been identified. Previous studies have also shown that in certain cases, low-level INH resistance correlates not with the loss of catalase activity but with the coacquisition of ETH resistance (*17*), indicating that the two drugs may share a common target.

We used a genetic strategy to identify the gene encoding the target of INH and ETH and to characterize mutations within the gene. A spontaneous INH-ETH–resistant mutant of M. *smegmatis*, mc^{2}651, was isolated from wt M. *smegmatis* mc^{2}155 in a single step with a mutational frequency of 10^{-7}. A genomic library from mc^{2}651 was constructed in a multicopy (5 to 10 copies) shuttle cosmid vector (9). A similar library was also made from M. *bovis* NZ (*18*), an INH-resistant mutant strain of M. *bovis*. Upon transfer of either library into wt M. *smegmatis* strains, cosmid clones were identified that consistently conferred INH-ETH resistance (Table 1). The transformation of cosmids containing a cross-hybridizing DNA fragment from wt (INH-ETH–sensitive) strains of M. *smegmatis*, M. *tuberculosis*, M. *bovis*, M. *bovis* BCG, and M. *avium* yielded clones that conferred INH-ETH resistance. We speculated that the INH-ETH resistance conferred by the transfer of the wt DNA fragment was due to overexpression of the target, as is the case for the resistance phenotype seen with several antibiotics (*13, 19*).

A 3-kb Bam HI DNA fragment from the M. *smegmatis* cosmid pYUB286 that conferred INH resistance was used as a probe for Southern (DNA) analysis. This probe strongly hybridized to all of the 11 different mycobacterial species tested, including the pathogenic strains M. *tuberculosis*, M. *bovis*, M. *avium*, and M. *leprae* (*20*), demonstrating that this sequence is highly conserved among the mycobacteria.

The DNA fragments that conferred resistance to INH were isolated from the wt (INH-sensitive) strains of M. *smegmatis*, M. *bovis*, and M. *tuberculosis*, as well as from the INH-resistant mutants of M. *smegmatis* and M. *bovis*. Sequence analysis (*21*) revealed that each strain contains two open reading frames (ORFs), one encoding a 29-kD protein followed by another encoding a 32-kD protein (Fig. 1). Subcloning studies demonstrated that the second ORF from M. *smegmatis* DNA was sufficient to confer the INH resistance phenotype and was thus named the *inhA* gene. In contrast to the M. *smegmatis* gene, the M. *tuberculosis* and M. *bovis inhA* genes appear to be in an operon with the gene encoding the 29-kD ORF, an observation confirmed by subcloning (Fig. 1). In M. *tuberculosis* and M. *bovis* DNA, the noncoding region between the two ORFs was substantially

Table 1. List of *inhA* genes from different mycobacteria that confer resistance to INH and ETH in *M. smegmatis* mc^{2}155 (*33*).

Strain	Plasmid	Description of plasmid	Source of insert	MIC (μg/ml)	
				INH	ETH
M. smegmatis mc^{2}155	—	—	—	5	10
M. smegmatis mc^{2}144	pYUB18	Vector	—	5	10
M. smegmatis mc^{2}845	pYUB314	pYUB18::*inhA*	*M. smegmatis* mc^{2}155	60	>80
M. smegmatis mc^{2}846	pYUB286	pYUB18::*inhA*	*M. smegmatis* mc^{2}651	60	>80
M. smegmatis mc^{2}802	pYUB315	pYUB18::*inhA*	*M. tuberculosis* H37R$_v$	15	30
M. smegmatis mc^{2}799	pYUB316	pYUB18::*inhA*	*M. bovis* BCG	15	30
M. smegmatis mc^{2}847	pYUB370	pYUB18::*inhA*	*M. bovis* NZ	30	60
M. smegmatis mc^{2}832	pYUB317	pYUB18::*inhA*	*M. avium*	60	>80
M. smegmatis mc^{2}651	—	—	—	50	>80
M. avium	—	—	—	5	10
M. tuberculosis H37R$_v$	—	—	—	~0.2	~10
M. bovis BCG	—	—	—	~0.2	~10

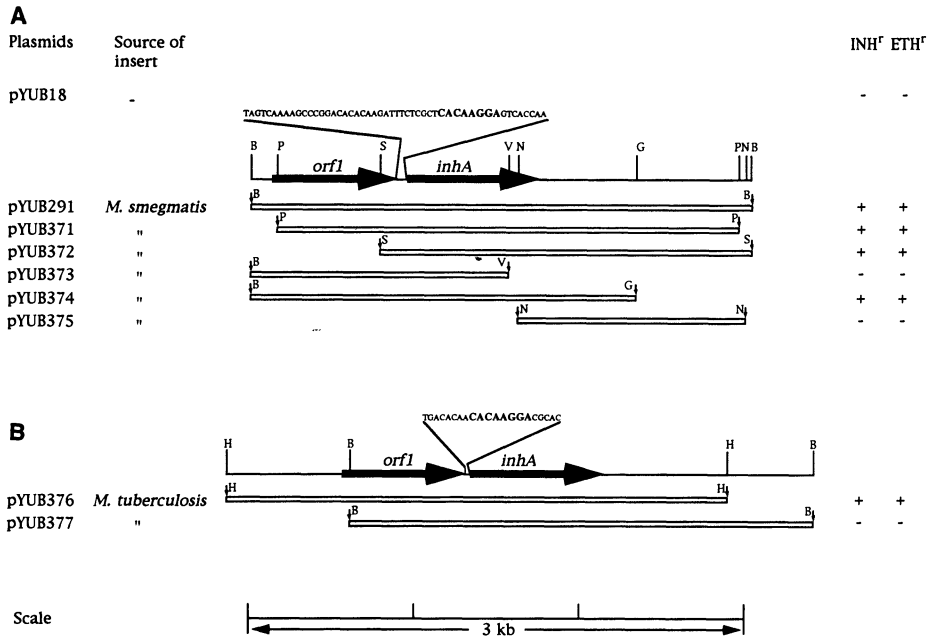

Fig. 1. Subcloning analysis to determine the smallest DNA fragments from (**A**) *M. smegmatis* mc^{2}155 and (**B**) *M. tuberculosis* H37R$_v$ that are sufficient to confer resistance to INH and ETH. The *M. smegmatis* mc^{2}155 were transformed with a pool of *E. coli*—mycobacteria shuttle cosmids (*28*), and individual clones were scored for resistance (r, +) or sensitivity (−) to INH and ETH. The ORF preceding *inhA* is labeled *orf1* and the sequence of the intervening DNA is shown. The ribosome binding sites are indicated in boldface letters. The following enzymes were used for subcloning: B, Bam HI; P, Pst I; S, Spe I; V, Pvu II; N, Nla III; G, Bgl II; H, Nhe I. All the subclones were tested in both orientations. Subcloning analysis of *M. bovis* DNA yielded results similar to those obtained with *M. tuberculosis* (*18, 20*). Plasmid YUB291 was also shown to confer INH and ETH resistance in *M. bovis* BCG host (*20*).

shorter than that in *M. smegmatis* and may lack a promoter that appears to be present in the latter strain (Fig. 1). The predicted InhA proteins of *M. tuberculosis*, *M. bovis*, and *M. smegmatis* show strong sequence similarity (40% identity over 203 amino acids) to the EnvM proteins of *Salmonella typhimurium* and *Escherichia coli* (Fig. 2). The protein EnvM is thought to be involved in fatty acid biosynthesis (*19*). The first ORF (*orf1*) also exhibits sequence similarity to several other proteins involved in fatty acid biosynthesis; the highest identity score was 46.5% over 241 amino acids of the 3-ketoacyl-acyl carrier protein reductase (*fabG* gene product) from *E. coli* (*20*). The InhA protein may use nicotinamide or flavin nucleotides as substrates or cofactors, as it has a putative binding site for these molecules (*22*).

The similarities of InhA and the ORF1 to proteins associated with fatty acid biosynthesis are consistent with the hypothesis that INH inhibits an enzyme involved in the biosynthesis of mycolic acid (long-chain fatty acids of the mycobacterial cell wall). Compared to wt extracts, cell-free extracts from the resistant mutant mc^{2}651 or from resistant merodiploids containing multiple copies of *inhA* showed marked resistance to the INH-mediated inhibition of mycolic acid biosynthesis (Fig. 3). This result supports the idea that InhA is required for mycolic acid biosynthesis.

The InhA protein from the INH-resistant mutant (mc^{2}651) differs from the wt (mc^{2}155) InhA by a single substitution of Ser to Ala at position 94 (Fig. 2). To test whether this difference caused the INH resistance phenotype in mc^{2}651, we performed an allele exchange experiment on the *M. smegmatis* chromosome. The mc^{2}651 cells were transformed with a 45-kb *M. smegmatis* DNA fragment that contained the wt *inhA* gene linked to a *kan*^{r} marker gene (*23*). Kanamycin-resistant transformants were screened for their sensitivity to INH. This sensitivity cotransformed with the kanamycin-resistance phenotype in 72% of the transformants, indicating that the drug sensitivity results from the replacement of the mutant *inhA1* allele by the wt *inhA* allele. Allelic exchange was confirmed by restriction fragment length polymorphism analysis of the *inhA* polymerase chain reaction (PCR) products obtained from the recombinants and by Southern blots (*20, 24*). Thus, the mutation of Ser to Ala^{94} mediates the INH-resistance phenotype in *M. smegmatis*. The INH-resistant mutant *M. bovis* NZ has an identical substitution of Ser to Ala (Fig. 2). The allelic exchange could not be done in *M. bovis* because there is no homologous recombination system available (*25*). However, the mutant *M. bovis* gene conferred a higher level of resistance to INH (100% survival in 20 μg of INH per milliliter, MIC = 30 μg/ml) than did the wt *M. bovis* gene (0% survival in 20 μg of INH per milliliter, MIC = 15 μg/ml) when transformed into *M. smegmatis* mc^{2}155 on a pYUB18 cosmid vector (Table 1). This result demonstrates that the mutation of Ser to Ala caused INH resistance in *M. bovis* NZ.

The *inhA* gene from INH-sensitive *M. tuberculosis* is 100% identical with the *inhA* gene of INH-sensitive *M. bovis* (*21*). Given that the mutation of Ser to Ala conferring INH resistance is conserved in *M. smegmatis* and *M. bovis*, we anticipate that some INH-resistant clinical isolates of *M. tuberculosis* will be found to carry missense mutations in the coding region of *inhA*. Alternatively, mutations that cause the overexpression of InhA (for example, in the *inhA* promoter or in a regulatory gene) or gene amplification could also mediate resistance.

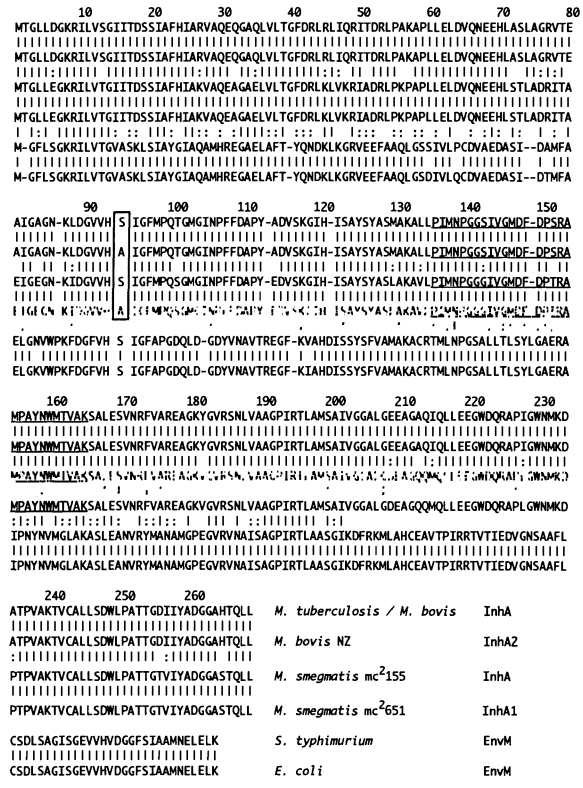

Fig. 2. Alignment of amino acid sequences of InhA proteins from *M. tuberculosis* H37R$_v$, *M. bovis*, *M. bovis* NZ, *M. smegmatis* mc^{2}155, and *M. smegmatis* mc^{2}651 with EnvM proteins from *S. typhimurium* and *E. coli*. The amino acid sequences were obtained by conceptual translation of the *inhA* and *envM* ORFs (*21*). Over a stretch of 203 amino acids, InhA and EnvM show ~75% sequence similarity (40% identity). InhA is highly conserved among mycobacterial strains. The InhA proteins of *M. tuberculosis* H37R$_v$ and *M. bovis* are identical and hence are represented by a single sequence. The *M. tuberculosis–M. bovis* InhA has >95% identity with the *M. smegmatis* InhA. The various *envM* gene products are also highly conserved (98% identity) (*19*). The position of the mutation conferring resistance to INH and ETH is indicated by the box. A putative binding site in InhA for nicotinamide or flavin nucleotide is underlined. InhA is the product of *inhA1*, the mutant allele of *inhA* isolated from the INH-resistant *M. smegmatis* mc^{2}651. InhA2 is the product of *inhA2*, the mutant allele of *inhA* isolated from the INH-resistant *M. bovis* NZ. Sequences were aligned with the GCG software package (*29, 30*). Identical residues are indicated by vertical lines and similar residues by dots. Gaps in alignment are indicated by hyphens.

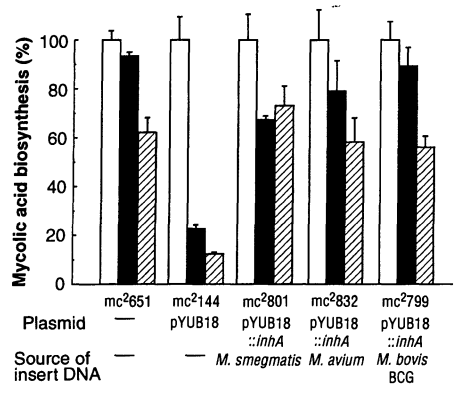

Fig. 3. Cell-free assays of mycolic acid biosynthesis. The *M. smegmatis* mc^{2}155 gene was transformed with pYUB18 vector (strain mc^{2}144) or pYUB18 carrying the *inhA* genes of *M. smegmatis* (pYUB291, product of subcloning of pYUB286, strain mc^{2}801), *M. avium* (pYUB317, strain mc^{2}832), or *M. bovis* BCG (pYUB316, strain mc^{2}799). Cell-free extracts were prepared from each of these strains and from the spontaneous INH-resistant mutant (mc^{2}651) of *M. smegmatis*. Incorporation of [1-^{14}C]acetate into mycolic acids was measured (*31*) after preincubation with or without INH. Protein concentrations in cell-free extracts were adjusted to 0.37 to 0.50 mg/ml, which resulted in the linear incorporation of radioactivity into the mycolic acids after a 1-hour incubation of the cell-free extract with [1-^{14}C]acetate. Each assay was done in duplicate, and the experimental error between different experiments was no more than 15%. The INH concentration necessary for strong inhibition of mycolic acid biosynthesis in cell-free extracts of the sensitive strain was about 20 times greater than the MIC (here, 20 × MIC = 100 μg/ml, solid bars.) Open bars, 0 μg/ml; crosshatched bars, 250 μg/ml. A 20- to 50-fold accumulation of INH has been reported to occur inside the mycobacteria (*16, 32*).

It is possible that mutations in genes other than *inhA* and *katG* may also cause INH resistance. Characterization of the full spectrum of mutations that confer resistance to INH could allow for the rapid identification of all INH-resistant strains by ligase chain reaction (*26*) or PCR single-strand conformational polymorphism strategies (*27*).

The ability to produce large quantities of the InhA protein will facilitate studies of the nature of InhA activity and the mode of action of INH and ETH in M. *tuberculosis*. Further investigation of this target may lead to the development of new agents for treating tuberculosis, including that caused by INH-resistant mutants of M. *tuberculosis* and disseminated M. *avium* infections, found frequently in late stage acquired immunodeficiency syndrome.

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23. A 45-kb-long DNA fragment containing the *inhA* allele from mc^{2}155 was cloned into a vector with Pac I sites flanking the insert, and a Tn*5*seq1 transposon (containing the *kan^r* gene) was introduced near *inhA* (*24*). The linear Pac I fragment containing *inhA* linked to *kan^r* was transformed into mc^{2}651 by electroporation. The transformants were plated on 7H10 plates containing kanamycin (15 μg/ml). The kanamycin-resistant transformants were then scored for INH sensitivity on 7H10 plates containing both kanamycin (15 μg/ml) and INH (10 μg/ml). INH sensitivity cotransformed with kanamycin resistance in 93 of 130 (72%) transformants tested. The remaining transformants were as resistant to INH as was mc^{2}651 (MIC = 50 μg/ml).
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30. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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33. Plasmid YUB18 (*28*) is a multicopy *E. coli*–mycobacteria shuttle cosmid. Cells of *M. smegmatis* mc^{2}155 (*28*) bearing the indicated plasmids were grown in 7H9 broth containing kanamycin (15 μg/ml), and dilutions were plated on 7H10 agar plates containing kanamycin alone or kanamycin with various concentrations of INH or ETH. The strains without any plasmid were grown in 7H9 broth, and dilutions were plated on 7H10 agar plates and on 7H10 agar plates with various concentrations of INH or ETH.

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Degeneration of a Nonrecombining Chromosome

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Comparative studies suggest that sex chromosomes begin as ordinary autosomes that happen to carry a major sex determining locus. Over evolutionary time the Y chromosome is selected to stop recombining with the X chromosome, perhaps in response to accumulation of alleles beneficial to the heterogametic but harmful to the homogametic sex. Population genetic theory predicts that a nonrecombining Y chromosome should degenerate. Here this prediction is tested by application of specific selection pressures to *Drosophila melanogaster* populations. Results demonstrate the decay of a nonrecombining, nascent Y chromosome and the capacity for recombination to ameliorate such decay.

Once the Y chromosome stops recombining with the X chromosome, it becomes an asexual (nonrecombining) component in an otherwise sexual genome (*1*, *2*). Asexual transmission of a chromosome leads to increased amounts of sampling error–induced linkage disequilibrium in finite populations. This interferes with the process of purifying selection and thereby causes chronic accumulation of small effect, deleterious mutations by a variety of mechanisms (*3*–*9*). The rate of accumulation increases with increasing mutation rate (U, the average per locus mutation rate multiplied by the number of loci per chromosome) and decreasing effective population size (N_e, essentially the number of successfully reproducing individuals). Population genetic theory predicts that recombination can stop or substantially slow the decay process (*4*–*9*). Studies have demonstrated that mutations accumulate when populations are repeatedly propagated through bottlenecks of a single individual (*10*–*12*). This report analyzes the effect of recombination on a simulation of a primitive Y chromosome without the use of recurrent single individual bottlenecks, thereby permitting natural selection to operate continuously.

Drosophila melanogaster populations were used to test for mutation accumulation in large autosomal haplotypes made to segregate like nascent Y chromosomes, that is, being always heterozygous and transmitted without recombination from father to son. Two things

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