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# Genetic Evidence that InhA of *Mycobacterium smegmatis* Is a Target for Triclosan

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**Three *Mycobacterium smegmatis* mutants selected for resistance to triclosan each had a different mutation in InhA, an enoyl reductase involved in fatty acid synthesis. Two expressed some isoniazid resistance. A mutation originally selected on isoniazid also mediated triclosan resistance, as did the wild-type *inhA* gene on a multicopy plasmid. Replacement of the mutant chromosomal *inhA* genes with wild-type *inhA* eliminated resistance. These results suggest that *M. smegmatis* InhA, like its *Escherichia coli* homolog FabI, is a target for triclosan.**

Triclosan is an antimicrobial agent (5, 9) which has been added to hand soaps, fabrics, plastics, and toothpastes, among other products. We have recently shown that triclosan inhibits lipid synthesis in *Escherichia coli*, with the probable target being enoyl reductase (FabI) (12), an essential enzyme which uses NADH to reduce a double bond during each cycle of fatty acid elongation (6). A subsequent confirmatory study also showed that purified FabI is inhibited by triclosan (10). The present work investigates whether targeting of the enoyl reductase InhA might explain the activity of triclosan (18) against *Mycobacterium smegmatis*.

The *inhA* locus was originally identified in *M. smegmatis* by a mutation (S94A, replacing serine 94 with alanine in the protein) which caused resistance to the antituberculosis drug isoniazid (3). The InhA protein of *M. smegmatis* is an enoyl reductase (7, 15) and is 35% identical to *E. coli* FabI (GAP program of Genetics Computer Group, Inc. [GCG]). It is 87% identical to *Mycobacterium tuberculosis* InhA, the three-dimensional structure of which has been determined by X-ray crystallography (7) in the presence of modified isoniazid (16). X-ray crystallography of *E. coli* FabI (2) demonstrates its structural similarity to InhA.

**Isolation and characterization of mutants of *M. smegmatis* selected for resistance to triclosan or to isoniazid.** *M. smegmatis* mc$^2$155 (from W. R. Jacobs, Jr.) was grown in LB broth (10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter) or 7H9 medium (see legend to Table 1) to stationary phase, and approximately $10^8$ CFU was plated onto LB agar (without Tween 80 or glycerol) containing 0.8 to 1.6 μg of triclosan (a trichlorinated diphenyl ether, from Ciba-Geigy Corp., Greensboro, N.C.) ml$^{-1}$. The largest of the 20 to 200 colonies of various sizes which appeared on each plate after 3 days were selected. Three independent mutants, MT1, MT9, and MT17, were chosen for study. Each was four to six times more resistant to triclosan than the parental strain (Table 1). Mutant MT1 manifested considerable resistance to isoniazid, MT17 showed less, and MT9 showed none (Table 1). Mutant mc$^2$651 (from W. R. Jacobs, Jr.), which has the S94A substitution in InhA (3), showed high isoniazid resistance, as expected. In addition, it had a triclosan resistance four to six times that of the wild type (Table 1). The wild-type *M. smegmatis inhA* gene on multicopy plasmid pMD31::*inhA*$^+$ (an unpublished Kan$^r$ *E. coli*-mycobacterium shuttle plasmid derived by subcloning a 3-kb *Bam*HI fragment including *orf1-inhA-orf3* into pMD31 [8]; a gift of L. Miesel) caused resistance to triclosan and isoniazid (Table 1), likely related to target overexpression. These data suggested that the *M. smegmatis* InhA is a target for triclosan.

**Substitution of wild-type *inhA* for mutant *inhA*.** If a mutation in *inhA* is responsible for both triclosan and isoniazid resistances, homologous replacement of the mutant *inhA* chromosomal gene with a wild-type *inhA* gene would eliminate the resistances. The method used pYUB325 (13) (from W. R. Jacobs, Jr.), a shuttle cosmid containing a large *Pac*I restriction fragment from the mc$^2$155 genome. Within this fragment are the wild-type *inhA*$^+$ gene and a nearby kanamycin resistance gene insert. pYUB325 (prepared from *E. coli* host STBL-2 [Gibco/BRL]) was digested with *Pac*I and extracted with phenol-chloroform. Chilled logarithmic-phase cells were pelleted at 4°C and resuspended gently in 0.2 volume of cold 10% glycerol–0.1% Tween 80, and 10% glycerol was added up to 1 volume. After another wash, the cells were resuspended in 0.01 volume of glycerol–Tween 80, and electroporation was performed (0.1 ml of cells, 0.2 μg of DNA, 0.2-cm cuvettes, 2.5 kV, 25 μF, 1,000 Ω). Then 1 ml of LB broth–0.5% Tween 80 was added, and the cells were grown for 4 to 16 h, plated on LB agar containing 15 μg of kanamycin ml$^{-1}$, and incubated 4 to 6 days.

Four kanamycin-resistant transformants of each mutant were assayed for drug susceptibility by agar dilution. All four transformants of mutant MT9, three transformants of MT1, three transformants of mc$^2$651, and one transformant of MT17 had lost both triclosan resistance and any isoniazid resistance that the parental strain possessed. The rest retained the mutant resistance phenotype. These results are compatible with the expected frequency of 30 to 70% for coinheritance of *inhA*$^+$ and Kan$^r$ (13). Therefore it is clear that the mutant *inhA* gene, or a gene very closely linked to it, was responsible for both triclosan and isoniazid resistance.

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TABLE 1. Characteristics of strains of *M. smegmatis*[a]

| Strain | Characteristic (reference) | InhA mutation | Relative MIC (SD)[b] of: | | |
|---|---|---|---|---|---|
| | | | Triclosan | | Isoniazid in 7H9 |
| | | | In LB | In 7H9 | |
| $mc^2155$ | Wild type (see reference 12) | None | 1.0 | 1.0 | 1.0 |
| MT1 | $mc^2155$ selected on triclosan (this work) | M161V | 4.9 (0.9) | 6.3 (2.0) | 8.5 (2.5) |
| MT9 | $mc^2155$ selected on triclosan (this work) | M103T | 4.4 (1.1) | 6.3 (2.0) | 1.2 (0.5) |
| MT17 | $mc^2155$ selected on triclosan (this work) | A124V | 4.0 (1.2) | 5.8 (1.7) | 2.0 (0.7) |
| $mc^2651$ | $mc^2155$ selected on isoniazid (3) | S94A | 4.4 (1.3) | 6.3 (2.0) | 22 (12) |
| $mc^2155$/pMD31::*inhA*$^+$ | $mc^2155$ bearing multicopy *inhA*$^+$ (see text) | None | 4.6 (0.6) | 6.3 (2.0) | >64 |

[a] All MICs were determined on agar plates by twofold serial dilutions using logarithmic-phase cells as described elsewhere (12). Cells were grown with 0.05% Tween 80 either in LB broth or in 7H9 medium supplemented with Middlebrook ADC enrichment (Difco) plus 0.2% glycerol and were tested on the corresponding solid medium without Tween 80. All plates with triclosan also contained 0.1% ethanol. Less clumping of cells during growth was seen in 7H9 than in LB, but the MIC for mutants on 7H9 agar approached the solubility limit of triclosan in this medium (50 to 100 μg ml$^{-1}$, observed visually).

[b] Results are means (± standard deviations) from four to five experiments. MICs are expressed as ratios to the MICs for $mc^2155$. MICs for $mc^2155$ are as follows (in micrograms per milliliter): triclosan in LB, 0.61 ± 0.15; triclosan in 7H9, 14 ± 5; isoniazid in 7H9, 7 ± 2.

**DNA sequence of *inhA* gene from mutants.** We sequenced the *inhA* gene in each of the three triclosan-selected mutants. Chromosomal DNA was prepared as described elsewhere (1), with a 2-h preliminary incubation at 37°C of cells with 4 mg of lysozyme ml$^{-1}$. PCR of the entire *inhA* gene was performed for each mutant by using *Taq* DNA polymerase (Gibco/BRL) at 2 mM $Mg^{2+}$ in EasyStart reaction tubes (Molecular Bio-Products). Primers LM026 (forward; 5′-AAAGCCCGGACACAC AAGA-3′) and LM027 (reverse; 5′-CGAACGACAGCAGTA GCAAG-3′) were chosen from sequences bracketing *inhA* (see GenBank accession no. I73544) by using the PRIME program of GCG and were annealed at 52°C. Both strands of the resulting 890-bp PCR product were sequenced (Tufts Core Facility) by using the same two primers.

The *inhA* structural gene of each mutant differed by a single nucleotide from the wild-type sequence (GenBank accession no. U02530). Together with the other results, this finding proved that a mutated *inhA* gene was responsible for the triclosan resistance in each mutant. Mutant MT1 had a replacement of methionine 161 (ATG) by valine (GTG). A mutation at the same residue of *E. coli* FabI (methionine 159) also causes triclosan resistance (12). Mutant MT9 had a replacement of methionine 103 (ATG) by threonine (ACG), and mutant MT17 had a replacement of alanine 124 (GCG) by valine (GTG).

**Discussion.** All three of the *M. smegmatis* InhA residues that were mutated in the present study, like those in FabI of triclosan-resistant *E. coli* (12), lie close to the NADH cofactor and putative acyl substrate binding sites (observed by using the program STING [14] with *M. tuberculosis* InhA [Protein Data Base 1ENY]; STING uses atomic coordinates to present a three-dimensional "virtual" protein image which can be manipulated). This supports the concept that InhA is the actual triclosan target. Like isoniazid (16) and diazaborine (2), triclosan might bind covalently to NADH. Resistance then might be explained, as for isoniazid (4, 7, 16), by reduced binding of NADH to the enzyme. Alternatively, the hydrophobic triclosan might bind noncovalently to the protein and interfere, directly or allosterically, with optimal binding of NADH or fatty acyl substrate in the active site. Mutations causing resistance would then prevent either the binding of triclosan or the allosteric response. Steric interference with the binding of the inhibitor diazaborine to the putative fatty acyl substrate binding site of *E. coli* FabI has been suggested as the resistance mechanism for a G93S mutation (2).

*M. smegmatis* is susceptible to triclosan, whereas *M. tuberculosis* is not (18). The four residues in *M. smegmatis* InhA which influence triclosan resistance, S94, M103, A124, and M161, are conserved in *M. tuberculosis*. They would not, therefore, identify any residues unique to *M. tuberculosis* InhA which might account for the intrinsic resistance. On the other hand, the latter resistance may be due to mechanisms unrelated to InhA, such as the activity of an endogenous efflux pump(s) analogous to those which operate on triclosan in other organisms (11, 17).

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