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Overexpression of the D-Alanine Racemase Gene Confers Resistance to D-Cycloserine in *Mycobacterium smegmatis*

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D-Cycloserine is an effective second-line drug against *Mycobacterium avium* and *Mycobacterium tuberculosis*. To analyze the genetic determinants of D-cycloserine resistance in mycobacteria, a library of a resistant *Mycobacterium smegmatis* mutant was constructed. A resistant clone harboring a recombinant plasmid with a 3.1-kb insert that contained the glutamate decarboxylase (*gadA*) and D-alanine racemase (*alrA*) genes was identified. Subcloning experiments demonstrated that *alrA* was necessary and sufficient to confer a D-cycloserine resistance phenotype. The D-alanine racemase activities of wild-type and recombinant *M. smegmatis* strains were inhibited by D-cycloserine in a concentration-dependent manner. The D-cycloserine resistance phenotype in the recombinant clone was due to the overexpression of the wild-type *alrA* gene in a multicopy vector. Analysis of a spontaneous resistant mutant also demonstrated overproduction of wild-type AlrA enzyme. Nucleotide sequence analysis of the overproducing mutant revealed a single transversion (G→T) at the *alrA* promoter, which resulted in elevated β-galactosidase reporter gene expression. Furthermore, transformants of *Mycobacterium intracellulare* and *Mycobacterium bovis* BCG carrying the *M. smegmatis* wild-type *alrA* gene in a multicopy vector were resistant to D-cycloserine, suggesting that AlrA overproduction is a potential mechanism of D-cycloserine resistance in clinical isolates of *M. tuberculosis* and other pathogenic mycobacteria. In conclusion, these results show that one of the mechanisms of D-cycloserine resistance in *M. smegmatis* involves the overexpression of the *alrA* gene due to a promoter-up mutation.

The resurgence of tuberculosis has been characterized by the emergence of significant numbers of drug-resistant strains. Furthermore, microorganisms of the *Mycobacterium avium* complex, opportunistic pathogens common in AIDS patients, are inherently resistant to many traditional antimycobacterial agents (20, 23). Hence, the development of novel drugs for the treatment of atypical infections by *M. avium*, *Mycobacterium intracellulare*, and multiple-drug-resistant *Mycobacterium tuberculosis* is urgently needed.

The mycobacterial cell wall is an effective barrier that contributes to drug resistance (45). Inhibitors of cell wall biosynthesis not only are potential antimycobacterial agents but also increase mycobacterial susceptibility to other antimicrobial agents (36). One inhibitor of cell wall synthesis is D-cycloserine (D-4-amino-isoxazolidone [DCS]), a cyclic structural analog of D-alanine (31). D-Amino acids, especially D-alanine, D-glutamate, and D-aminopimelate, are important components of all bacterial cell walls, including those of mycobacteria. Alanine is usually available as the L stereoisomer, and the conversion to D-alanine by the cytoplasmic enzyme D-alanine racemase (25) is required for the initial step in the alanine branch of peptidoglycan biosynthesis. D-Alanine is converted to the dipeptide D-alanyl–D-alanine in a reaction catalyzed by D-alanyl:alanine synthetase (D-alanine ligase [30]). In *Escherichia coli*, both D-alanine racemase and D-alanine ligase are targets of DCS (26, 31, 33). Moreover, the biosynthesis of mycolyl-arabinogalactan-peptidoglycan complex is inhibited by DCS in *M. tuberculosis* (10), and biochemical studies indicated that D-alanine ligase is one of the targets in mycobacteria (11).

DCS is an effective antimycobacterial agent but is rarely prescribed and used only in combined therapies due to its adverse effects (21, 22, 54). These side effects are due to binding of DCS to neuronal *N*-methyl aspartate receptors (44) and inhibition of enzymes that metabolize and synthesize the neurotransmitter γ-aminobutyric acid (53). Nevertheless, DCS is an excellent candidate for the development of a new generation of antibiotics. Two important considerations predict that rationally designed derivatives of DCS may be more efficacious antimicrobial agents. First, DCS targets participate in essential steps of cell wall synthesis. Second, DCS resistance has not yet become an important clinical problem. Therefore, the identification of DCS targets and the elucidation of the mechanisms leading to DCS resistance may contribute to the development of new therapeutics with fewer side effects and mechanisms of action which do not favor the emergence of resistance.

Few studies on the mode of action and mechanisms of DCS resistance in mycobacteria have been conducted. David (9) isolated and characterized step-wise DCS-resistant (DCS^r) mutants of *M. tuberculosis* and discovered mutants that showed either normal or reduced cellular permeability to DCS. It was hypothesized that mutants with normal uptake carried mutations in the D-alanine ligase gene, but no biochemical or molecular evidence in support of this hypothesis was provided.

Here we describe the first molecular genetic analysis of DCS resistance in mycobacteria, which led to the identification of one of the DCS targets and resistance mechanisms in *Mycobacterium smegmatis*. A spontaneous DCS^r mutant strain of *M. smegmatis* exhibited a promoter-up mutation in the D-alanine racemase gene (*alrA*) which increased the levels of expression

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli DH5α	*recA lacZ*Δ*M15*	Gibco-BRL
M. smegmatis mc^{2}155	High-transformation mutant of *M. smegmatis* ATCC 607	41
M. smegmatis GPM14[a]	*M. smegmatis* first-step DCS^r spontaneous mutant derived from mc^{2}155; overproduces D-alanine racemase	This work
M. smegmatis GPM16	*M. smegmatis* first-step DCS^r spontaneous mutant derived from mc^{2}155	This work
M. intracellulare mc^{2}76	Highly transformable *M. avium* complex strain	W. R. Jacobs, Jr., 16
M. bovis BCG	French isolate (Pasteur substrain)	W. R. Jacobs, Jr.
pCV77	Replicating *E. coli-Mycobacterium* shuttle plasmid; carries cassette of the promoterless *lacZ* gene with ribosome-binding site outflanked by transcriptional terminators	MedImmune Inc.
pMV203	Replicating *E. coli-Mycobacterium* shuttle plasmid; precursor of pMV262 without P_{hsp60}	MedImmune Inc.
pMV262	Replicating *E. coli-Mycobacterium* shuttle plasmid; carries P_{hsp60} promoter upstream from polylinker site	MedImmune Inc., 7
pYUB178	Integration-proficient shuttle cosmid vector; integrates at the attachment site of mycobacteriophage L5	34
pBUN19	pMV262 with the 3.1-kb insert from GPM16 in the *Bam*HI site	This work
pBUN25	pMV262 with the 0.9-kb *Pst*I fragment of pBUN19 in the *Pst*I site	This work
pBUN47D	pMV262 with the 2.0-kb *Pst*I fragment of pBUN19 in the *Pst*I site	This work
pBUN66	Recombinant plasmid isolated from an *M. smegmatis* mc^{2}155 cosmid library which hybridized with the 3.1-kb insert of pBUN19	24, W. R. Jacobs, Jr., this work
pBUN82	pMV262 with the 1.9-kb *Sca*I/*Hin*dIII fragment of pBUN19 in the *Pvu*II/*Hin*dIII site	This work
pBUN83	pMV262 with the 2.0-kb *Dra*I/*Cla*I fragment of pBUN19 in the *Dra*I*Cla*I site	This work
pBUN92	pMV203 with the 2.9-kb *Eco*RI/*Eco*RV fragment of pBUN19 in the *Eco*RI/*Hpa*I site	This work
pBUN101	pCV77 with the 0.5-kb fragment containing the upstream noncoding region of the *alrA* gene from mc^{2}155 inserted at the polylinker site	This work
pBUN102B	pCV77 with the 0.5-kb fragment containing the upstream noncoding region of the *alrA* gene from GPM14 inserted at the polylinker site	This work

[a] GPM, Great Plains Mycobacterial Collection.

of the gene and determined a DCS^r phenotype. Furthermore, transformants of *M. intracellulare* and *M. bovis* BCG with the *M. smegmatis alrA* gene carried in a multicopy vector had a DCS^r phenotype, indicating that a similar mechanism of resistance may occur in pathogenic mycobacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani broth or agar. *M. intracellulare* strains were grown as previously described (16). *M. smegmatis* strains were grown at 37°C with shaking (200 rpm; Innova 4300 incubator shaker; New Brunswick Scientific, Edison, N.J.) in Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, Md.), Luria-Bertani broth, or minimal medium (55) in the presence of 0.05% Tween 80. Tryptic soy agar base (Difco Laboratories, Detroit, Mich.) was used for growth of *M. smegmatis* on solid media. Spontaneous DCS-resistant *M. smegmatis* mutants were isolated by plating approximately 5.0×10^9 exponentially growing cells on tryptic soy agar with 500 to 600 μg of DCS ml^{-1}. Since DCS is moderately unstable, agar plates containing DCS were kept at 4°C for a maximum of 2 days. The independent mutants GPM14 and GPM16 were isolated at 500 and 600 μg ml^{-1}, respectively, from the susceptible parent strain mc^{2}155 (Fig. 1). To determine inhibition of colony formation, appropriate dilutions of exponentially growing cells (5.0×10^8 to 1.0×10^9 CFU ml^{-1}) were plated in triplicate onto agar containing a maximum of 1,500 μg of DCS ml^{-1} for *M. smegmatis* or up to 250 μg of DCS ml^{-1} for *M. intracellulare*. Regression analyses of bacterial titers at different DCS concentrations were conducted by using Proc Reg (SAS Institute, Cary, N.C.). The reduced model for the different bacterial strains was tested against the full model for lack of fit for each of the mycobacterial species tested. A significant lack of fit indicated that the responses to the DCS concentrations were different between the strains. If a significant lack of fit was determined when all the strains were considered, subsets of strains were also compared.

For D-alanine racemase assays, *M. smegmatis* cells were grown in minimal medium (55) to mid-exponential phase (ca. 3.0×10^8 CFU ml^{-1}). For β-galactosidase assays, the *M. smegmatis* strains were grown in Middlebrook 7H9 broth to mid-exponential phase (ca. 3.0×10^8 CFU ml^{-1}).

Transformation of *E. coli* and mycobacteria was carried out as described previously (16). *E. coli* and *M. intracellulare* transformants were selected at 50 μg of kanamycin ml^{-1}. *M. smegmatis* transformants were selected at either 10 μg of kanamycin ml^{-1} or 10 μg of kanamycin (Sigma Chemical Co., St. Louis, Mo.) ml^{-1} plus 300 μg of DCS (Sigma or Aldrich Chemical Co., Inc., Milwaukee, Wis.) ml^{-1}.

Oligonucleotides, PCR amplifications, and probe labeling. The oligonucleotide primers (Ransom Hill Biosciences, Inc., Ramona, Calif.) for PCR amplification of the D-alanine racemase genes from pBUN66 and GPM14 were JIM-1 (5′-GNGAYYNYGGRTACACCGAGTTC-3′) and JIM-2 (5′-CGNCGRCG AGCNNCTCGAAATC-3′). The oligonucleotide primer pair for probe labeling by PCR was NAN-1 (5′-TCTGCGGCCTCTGGGACAATGGG-3′) and NAN-2

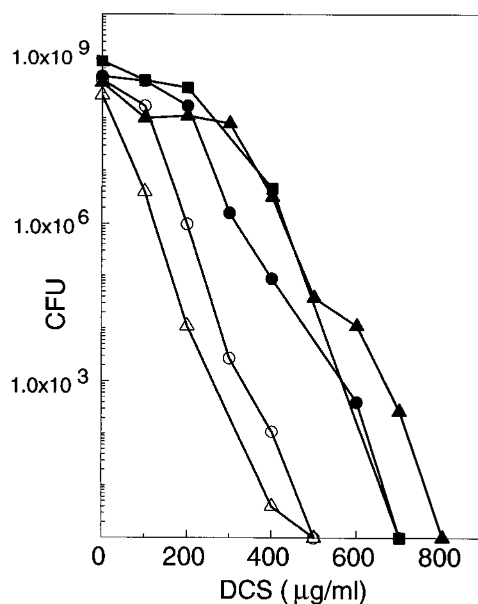

FIG. 1. Inhibition of CFU at increasing DCS concentrations for *M. smegmatis* mc^{2}155 (open circles), DCS-resistant mutant GPM14 (closed squares), DCS-resistant mutant GPM16 (closed circles), mc^{2}155(pMV262) (open triangles), and mc^{2}155(pBUN19) (closed triangles). The curves were generated from data from a representative experiment. For statistical analysis, performed for at least two independent experiments for each strain, see the text.

(5′-GACACACCTGCCACGGTGCCGAC-3′). The amplifications of the upstream, noncoding regions of the D-alanine racemase genes from mc^{2}155 and GPM14 were done with JIM-2 and JIM-3 (5′GTGTGGCGCAACAAAGAG-3′). PCR amplifications were carried out with *Taq* DNA polymerase (Fisher Scientific Co., Pittsburgh, Pa.) for 30 cycles in a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400; Roche Molecular Systems, Branchburg, N.J.) and required 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 0.2 mM spermidine, 10% (vol/vol) dimethyl sulfoxide, and 0.1 mg of gelatin ml^{-1} under standard cycling temperatures (hot start at 95°C, denaturation at 94°C, annealing at 55°C, and polymerization at 72°C). For radioactive labeling, 10 cycles were run in the presence of 50 μCi of [α-^{32}P]dATP. The Expand high-fidelity PCR system (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used as recommended by the manufacturer.

Nucleic acid manipulations, DNA sequencing, and primer extension analysis. For restriction digestions, ligations, agarose gel electrophoresis, and Southern hybridizations under stringent conditions, standard procedures were followed as previously described (40). Chromosomal DNA from *M. intracellulare* strains, *M. paratuberculosis*, and *M. smegmatis* was prepared as described previously (51). Chromosomal DNA from BCG and *M. tuberculosis* was provided by W. R. Jacobs, Jr., and T. Weisbrod.

Total RNA from mc^{2}155, GPM14, and GPM16 was isolated by following the procedure described by Bashyam and Tyagi (3). Northern blotting was done as previously described (1). Quantification of mRNA was carried out by capturing the images from the autoradiograms with a Kaiser RS-1 video camera and a Northern Light model 890 illuminator (Kaiser Optical Systems, Inc., Ann Arbor, Mich.) and analyzing the output with NIH Image 1.49 software. The levels of RNA were normalized by the amount of rRNA.

Sequencing reactions were carried out with the *Taq* DyeDeoxy FS terminator cycle sequencing kit (The Perkin-Elmer Corp., Norwalk, Conn.). The unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep; Princeton Separations, Adelphia, N.J.). The samples were dried, resuspended in loading buffer, heat denatured, and loaded in ABI model 377 DNA sequencers (Applied Biosystems, Foster City, Calif.). Each template was sequenced in its entirety in both orientations to prevent potential errors in sequencing. DNA sequencing and nucleotide sequence analyses were performed at the University of Minnesota's Advanced Genetic Analysis Center (St. Paul). Protein sequence analysis was performed with the Genetics Computer Group package (version 8.1), University of Wisconsin (13).

For the assessment of promoter strength by β-galactosidase reporter gene assays, 1.8-kb DNA fragments were amplified with primers JIM-2 and JIM-3, the products were digested with *Sca*I and *Cla*I, and the resulting 0.5-kb fragments were directionally cloned into the *Cla*I and the blunt-ended *Pst*I sites of pCV77. The synthesis of blunt-ended termini was carried out with *Pfu* DNA polymerase as instructed by the supplier (Stratagene, La Jolla, Calif.). All constructs were verified by sequencing of the relevant regions.

Primer extension analysis of *alrA* mRNA was carried out as described previously (12). The oligonucleotide NAN-3 (5′-ATCGATCACCGTCTGTGCCG ACGCC-3′) was radiolabeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega). The reactions were extended with Moloney murine leukemia virus reverse transcriptase (Promega). Radioactivity in primer extension bands was quantified with a PhosphorImager by using ImageQuant software version 3.3 (Molecular Dynamics, Sunnyvale, Calif.).

***M. smegmatis* genomic libraries.** An *M. smegmatis* mc^{2}155 cosmid library was kindly provided by W. R. Jacobs, Jr. For *M. smegmatis* GPM16 genomic libraries, chromosomal DNA was partially digested with *Sau*3A, and fragments with an average size of 3.0 kb were purified from a 0.8% agarose gel and ligated into the *Bam*HI site of the *E. coli-Mycobacterium* shuttle plasmid pMV262 (7), which carries the kanamycin-resistant marker and the strong promoter P_{hsp60}. The ligation mixture (approximately 1.0 μg of vector DNA) was transformed into *M. smegmatis* mc^{2}155. For selection of DCS resistance determinants, cells were plated on 10 μg of kanamycin ml^{-1} plus 300 μg of DCS ml^{-1} and yielded two DCS-resistant clones. Parallel platings of the transformation mixture on 10 μg of kanamycin ml^{-1} indicated a transformation efficiency of 4×10^4. Plasmids were isolated from independent kanamycin-resistant transformants and analyzed in *E. coli* for the presence of *M. smegmatis* inserts. This analysis revealed approximately 10% recombinant plasmids. Hence, the two DCS-resistant clones resulted from a representative library of ca. 4,000 recombinants.

Preparation of crude cell extracts. Cells were harvested and concentrated 20-fold in 50 mM Tris-HCl (pH 8.0). Cells were kept on a salt-ice-water bath and sonicated with a Vibra-Cell model VC600 disrupter (Sonic and Materials, Inc., Danbury, Conn.). Sonication was carried out for 10 min at 80% power output and 50% duty cycle, and in the presence of one-third the final volume of type A-5 alumina (Sigma). The resulting extracts were centrifuged at 4°C in a JA-17 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 30 min at 15,000 rpm, dialyzed against 50 mM Tris-HCl (pH 8.0), and sterilized by filtration through a 0.22-μm-pore-size filter. The protein concentration was determined by the *DC* assay (Bio-Rad Laboratories, Richmond, Calif.) as recommended by the manufacturer.

Enzyme assays. D-Alanine racemase activity in crude extracts was assayed in the direction of the conversion of L-alanine into D-alanine by a modification of the coupled spectrophotometric method described by Wijsman (52). Pilot experiments were performed to determine the amount of each extract and incubation times (15 min) resulting in a linear conversion of the substrate into the product. Crude cell extracts were incubated at 37°C in 1.0 ml each of reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 0.1 mM pyridoxal phosphate (Sigma), and 15 mM L-alanine (Sigma). To start the reactions, crude cell extracts were added to prewarmed mixtures. For inhibition assays, DCS was added at 10, 50, or 100 μg ml^{-1}. Reactions were terminated by boiling for 10 min. Subsequently, 0.25 mg of D-amino acid oxidase (Boehringer Mannheim), 0.2 mM NADH (Sigma), and 10 U of lactate dehydrogenease (Sigma) were added. The coupled reaction was measured by the change in absorbance at 340 nm after overnight incubation at 37°C. All controls and samples were measured in triplicate. For the calculation of specific activities, the background change in absorbance (obtained with boiling-inactivated extracts processed in an identical manner) was subtracted from the change in absorbance obtained with active extracts. From this net absorbance change (ΔA_{340}), the specific activity (in micromoles of L-alanine per minute per milligram) was calculated by using the following equation: $[\Delta A_{340}/t]/[6.2 \times C_P]$, where t is time (in minutes), 6.2 is the constant used to convert from A_{340} into micromoles (in milliliters per micromole, and C_P is protein concentration (in milligrams per milliliter of reaction mixture). No net change in absorbance was detected with active extracts when L-alanine was omitted from the reaction mixtures.

The β-galactosidase activity was determined in crude extracts as described previously (29). Pilot experiments were performed for each cell extract to determine the optimal incubation time and protein concentration resulting in a linear hydrolysis of β-*o*-nitrophenylgalactoside. Units of β-galactosidase activity per milligram were calculated as follows: (ΔA_{420} of sample $-$ ΔA_{420} of control) $\times$ $380/(t \times A_P)$, where 380 is the constant used to convert from A_{420} into β-galactosidase units. t is time (in minutes) at 28°C, and A_P is amount of protein (in milligrams) in the reaction mixture.

Nucleotide sequence accession number. Sequence data corresponding to the *M. smegmatis* D-alanine racemase gene cloned in pBUN19 and flanking sequences appear in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession no. U70872.

RESULTS

Cloning and characterization of *M. smegmatis* DCS resistance determinants. DCS^r mutants of *M. smegmatis* were isolated, and a genomic library of a mutant strain was constructed in a multicopy plasmid. By using this type of cloning strategy, either wild-type genes producing a DCS^r phenotype due to a gene dosage effect or genes with dominant mutations encoding proteins which are insensitive to drug inhibition can be isolated. However, this strategy does not readily identify mutations involved in DCS transport. In this work, two spontaneous DCS^r mutants, GPM14 and GPM16, were isolated at 500 and 600 μg of DCS ml^{-1}, respectively, from the DCS-sensitive (DCS^s) strain mc^{2}155 with a frequency of 1.0×10^{-9}. These DCS^r strains were identical to the parent strain with respect to generation time, colony morphology, phage susceptibility, and susceptibility to antimicrobial agents other than DCS, indicating that these mutants carry a mutation(s) specific for DCS resistance. The mutant strain GPM16 was selected for the construction of a genomic library.

The genomic library was constructed in *E. coli-Mycobacterium* shuttle plasmid pMV262, which replicates with a copy number of 5 to 10 in mycobacteria and carries the kanamycin resistance selection marker (7) (Table 1). The library was transferred to the DCS^s strain mc^{2}155 for the isolation of transformants resistant to kanamycin and DCS. Plating of a representative library (approximately 4,000 recombinants) led to the isolation of a recombinant plasmid (pBUN19) carrying a 3.1-kb insert. Retransformation of the DCS^s strain mc^{2}155 with this plasmid resulted in 100% of the transformants displaying a DCS^r phenotype, indicating that a DCS resistance determinant(s) was present in pBUN19.

The DCS^r phenotypes of mutants, recombinants, and controls were characterized by the inhibition of colony formation at increasing DCS concentrations (Fig. 1). The control group, strains mc^{2}155 and mc^{2}155(pMV262), had approximately the same susceptibility to DCS (P = 0.80). Strains GPM14,

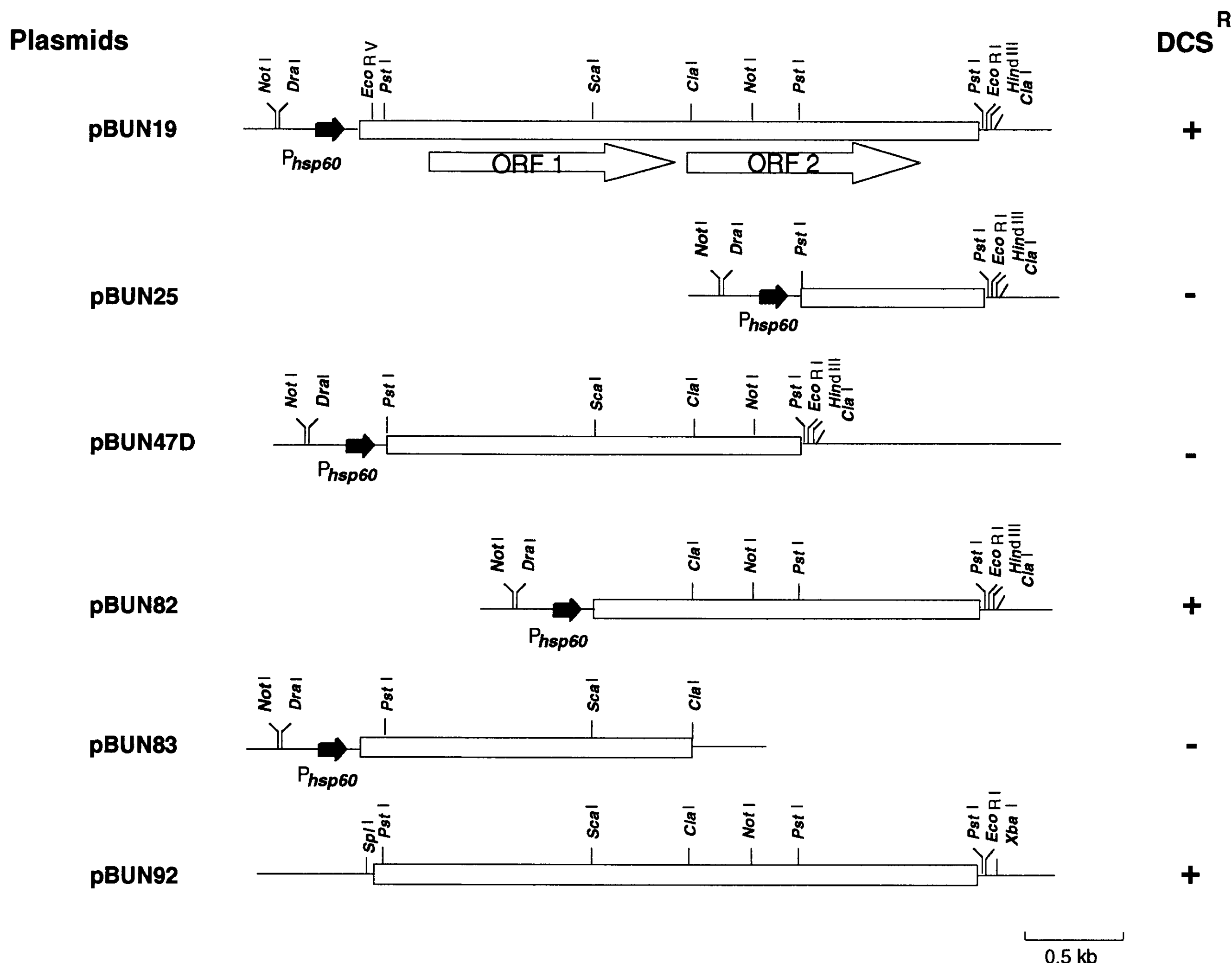

FIG. 2. Subcloning analysis of pBUN19 and the DCS-resistant phenotype. The position of the P_{hsp60} promoter in vector pMV262 is indicated. *M. smegmatis* sequences (open boxes) and the locations of ORFs are shown. The DCS resistance phenotypes of corresponding subclones are indicated by plus (resistant) and minus (sensitive) signs.

GPM16, and mc^{2}155(pBUN19) were significantly more resistant than the control group ($P < 0.00001$).

ORF and subcloning analysis of the DCS resistance determinant. Nucleotide sequence analysis of the 3,059-bp DNA insert from pBUN19 revealed two open reading frames (ORFs) (Fig. 2). ORF1 (1,236 nucleotides) has significant homology with the *E. coli* glutamate decarboxylase gene, (*gadS* [28], or *gadA* [42]). ORF2 (1, 167 nucleotides) encodes a product with significant homology to D-alanine racemases from several microbial species. The *M. smegmatis* D-alanine racemase gene and its immediate flanking regions were sequenced. An initiation codon (ATG) at position 51 and a putative ribosomal binding site (GAGAT) separated from the initiation codon by 7 bp were identified. Subcloning experiments were performed to further localize the DCS^r determinant within the 3.1-kb insert of pBUN19. The *Sca*I/*Hin*dIII DNA fragment subcloned in pBUN82, which contains the complete D-alanine racemase gene (*alrA*) but only a truncated glutamate decarboxylase gene (*gadA*), was sufficient for a DCS^r phenotype (Fig. 2). In contrast, subcloning of the 2.0-kb *Dra*I/*Cla*I fragment in plasmid pBUN83, which contains the *gadA* gene, gave a DCS^s phenotype. Furthermore, subcloning of DNA fragments into pMV262, which split *alrA* (plasmids pBUN25 and pBUN47D), resulted in DCS^s transformants (Fig. 2). Hence, *alrA* is necessary and sufficient to confer DCS^r to mc^{2}155.

The presence of homologous *alrA* alleles in pathogenic mycobacteria was tested by Southern analysis with the D-alanine racemase gene amplified by PCR as a probe (Fig. 3). This probe hybridized under stringent conditions with chromosomal DNA from *M. smegmatis*, *M. intracellulare* mc^{2}76, *Mycobacterium paratuberculosis* ATCC 19698, *M. bovis* BCG, and *M. tuberculosis*. Digestion of *M. smegmatis* mc^{2}155 DNA with *Bam*HI, *Eco*RI, and *Hin*dIII yielded single bands of 7.3, 10.8, and 20 kb, respectively. Digestion with *Pst*I, which cuts once within *M. smegmatis alrA*, revealed two bands of 6.4 and 1.2 kb in *M. smegmatis*, single bands of 7.4 and 5.5 in *M. intracellulare* and *M. paratuberculosis*, and bands of 9.5, 7.1, and 0.9 kb in strains of the *M. tuberculosis* complex. The banding pattern of the *M. smegmatis* DNA *Pst*I digest differed from the pattern obtained with the recombinant plasmid pBUN19, which possesses a noncontiguous *Sau*3A DNA fragment (ca. 0.5 kb) upstream from *alrA* which carries a *Pst*I site.

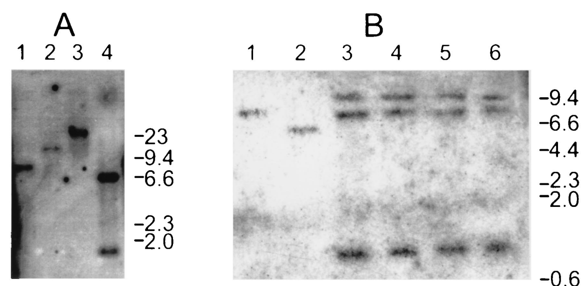

FIG. 3. (A) Southern blot of total DNA from *M. smegmatis* digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), and *Pst*I (lane 4). (B) Southern blot of total DNA from different mycobacterial species digested with *Pst*I: *M. intracellulare* mc^{2}76 (lane 1), *M. paratuberculosis* ATCC 19698 (lane 2), *M. bovis* BCG-Pasteur (lane 3), and *M. tuberculosis* H37R$_a$ (lane 4), H37R$_v$ (lane 5), and Erdman (lane 6). Blots were hybridized with the radiolabeled 1,092-bp PCR fragment from pBUN19 under stringent conditions as indicated in Materials and Methods.

Sequence analysis of the *M. smegmatis* D-alanine racemase (*alrA*) gene. The alignment of the inferred amino acid sequences of D-alanine racemases from several bacterial species is displayed in Fig. 4. The predicted 41.0-kDa polypeptide displayed 66% amino acid identity to the homologous predicted polypeptides in *M. leprae* (17) (accession no. U00020) and *M. tuberculosis* (35) (accession no. Z77165). The *M. smegmatis* polypeptide has approximately 35% identity to the D-alanine racemase isozymes from *Bacillus stearothermophilus* (2, 43), *Bacillus subtilis* (14), *E. coli* (5, 27), and *Salmonella typhimurium* (49, 50) and the putative enzyme from *Haemophilus influenzae* (15). Multiple amino acid sequence alignments showed highly conserved domains: the amino acid sequence A^{66}VVKANAYGHG^{76} in the consensus, which contributes to the pyridoxal phosphate binding domain in the active site (18), and the conserved lysine which covalently binds pyridoxal phosphate in the catalytic cycle (2, 39). All the mycobacterial D-alanine racemases display a two-domain structure, as observed in the isozymes from *S. typhimurium* (19). In this larger alignment, the consensus at the hinge region that links the two domains is defined as V^{302}-YG--W^{308}.

Analysis of D-alanine racemase activity and the inhibitory effect of DCS in *M. smegmatis*. We determined the D-alanine racemase specific activity of cell extracts from the parent strain, i.e., mc^{2}155, mutant GPM16, and recombinant strain mc^{2}155 (pBUN19) (Fig. 5). The D-alanine racemase activity in each cell extract was inhibited by DCS in a concentration-dependent manner. Degrees of inhibition by DCS were similar for all these strains (data not shown). These results confirmed that D-alanine racemase is one of the drug targets. Surprisingly, the mutant GPM16 displayed levels of D-alanine racemase and a pattern of DCS inhibition similar to that of the wild-type strain. Therefore, its resistance phenotype appears to be due to a mutation in a separate DCS resistance determinant, distinct from the D-alanine racemase gene. In contrast, the recombinant strain had a 15-fold-higher specific activity. We hypothesized that the resistance phenotype in the recombinant strain was the consequence of elevated expression of the wild-type D-alanine racemase gene harbored in the multicopy vector. To verify this hypothesis, a cosmid library of the wild-type strain mc^{2}155 was screened with the objective of identifying and analyzing the D-alanine racemase gene. The recombinant plasmid pBUN66 was isolated by colony hybridization with the *Dra*I-*Hin*dIII fragment from pBUN19 as a probe (see Materials and Methods). The wild-type D-alanine racemase nucleotide gene and flanking sequences in pBUN66 were PCR amplified and sequenced. The D-alanine racemase gene in pBUN66 was identical to the one cloned in pBUN19, confirming that the mutant GPM16 had a wild-type D-alanine racemase gene. Therefore, this result together with the biochemical data strongly suggests that the DCS-resistant phenotype of the recombinant strain mc^{2}155(pBUN19) was due to an overexpression of the D-alanine racemase gene and not a mutation in the structural gene. Furthermore, the recombinant plasmid pBUN92 (Fig. 2), which does not carry the P_{hsp60} promoter, exhibits a DCS^r phenotype as well. Hence, multiple copies of the wild-type D-alanine racemase gene appear to cause the DCS^r phenotype of the recombinant strain. In agreement with the *E. coli* (5) (accession no. U00006) and *M. leprae* (17) (accession no. U00020) designations, we propose the designation *alrA* for the *M. smegmatis* D-alanine racemase gene, which is constitutively expressed (6).

Overproduction of D-alanine racemase is also a mechanism of resistance in spontaneous *M. smegmatis* DCS^r mutants. Since the observation of the overproduction of D-alanine racemase in the recombinant strain mc^{2}155(pBUN19) was the result of a laboratory manipulation, we analyzed four additional DCS^r independent mutants derived from mc^{2}155. The crude extract of one of these mutants, GPM14, displayed levels of D-alanine racemase specific activity similar to those displayed by the recombinant strain mc^{2}155(pBUN19) (Fig. 5). The spontaneous DCS^r mutant GPM14 exhibited approximately 20-fold-greater D-alanine racemase specific activity than the DCS^s strain mc^{2}155. The D-alanine racemase activity of GPM14 was inhibited by DCS in a fashion similar to that of the wild-type enzyme. In agreement with the enzyme activity assays, Northern blot analysis demonstrated a 30-fold overexpression of D-alanine racemase *alrA* mRNA in GPM14 compared to that in strain mc^{2}155 (data not shown).

A promoter-up mutation leads to the overexpression of the D-alanine racemase (*alrA*) gene in *M. smegmatis* DCS^r mutant GPM14. The D-alanine racemase allele from GPM14 was PCR amplified and sequenced. Nucleotide sequence analysis did not show any mutation within the structural gene but revealed a single base change, T for G, in the upstream noncoding regions. The identification of the DCS^r mutant GPM14, which possessed an elevated level of D-alanine racemase specific activity but an unaltered D-alanine racemase structural gene, established solid evidence that overproduction of this enzyme could occur by a natural mechanism.

Mapping of the mRNA start site in mc^{2}155 and GPM14 was carried out by primer extension analysis (Fig. 6). In each case, two start sites, one nucleotide apart, were found 13 and 14 bp upstream from the amino acid start codon, with the product of the shorter sequence being more abundant. The point mutation in GPM14 was located within the putative −10 promoter

FIG. 4. Multiple sequence alignment of bacterial D-alanine racemases that exhibit similarity to the *M. smegmatis* ORF2 DCS resistance determinant from pBUN19 (Msmegm). Black boxes indicate complete identity, and shaded boxes indicate conservative amino acid substitutions. Sequences were obtained from GenBank. The sequences are from various organisms as follows: Bsth-Cat, *B. stearothermophilus* (catabolic isozyme; accession no. M19142 [43]); Bsubt, *B. subtilis* (accession no. M16207 [14]); Mleprae, *M. leprae* (accession no. U00020 [17]); Mtb, *M. tuberculosis* (accession no. Z77165 [35]); Ecoli-Cat, *E. coli* (catabolic isozyme; accession no. L02948 [27]); Styph-Cat, *S. typhimurium* (catabolic isozyme; accession no. K02119 [49]); Styph-Bio, *S. typhimurium* (biosynthetic isozyme; accession no. M12847 [18]); Ecoli-Bio, *E. coli* (biosynthetic isozyme; accession no. U00006 [5]); and Hinf-Bio, *H. influenzae* (biosynthetic isozyme; accession no. L46206 [15]).

```
 Bsth-Cat .......... .......... .......... MNDFHRDTWA EVDLDAIYDN VENLR  25
    Bsubt .......... .......... ........MS TKPFYRDTWA EIDLSAIKEN VSNMK  27
  Mleprae .......... .......... ..MAVTPISL RPGVLAEA.. VVDLGAIDYN VRVLR  31
      Mtb VKRFWENVGK PNDTTDGRGT TSLAMTPISQ TPGLLAEA.. MVDLGAIEHN VRVLR  53
   Msmegm .......... .......... ..MQTTEPMT PPAPLASAQT VIDLGAIDHN VRVLR  33
Ecoli-Cat .......... .......... .......... ...MTRPIQA SLDLQALKQN LSIVR  22
Styph-Cat .......... .......... .......... ...MTRPIQA SLDLQVMKQN LAIVR  22
Styph-Bio .......... .......... .......... ....MQAATV VINRRALRHN LQRLR  21
Ecoli-Bio .......... .......... .......... ....MQAATV VINRRALRHN LQRLR  21
 Hinf-Bio .......... .......... .......... ..MNVKPATA KISSHALKQN LEIIK  23
Consensus ---------- ---------- ---------- -------A-- --DL-A---N ---LR  55

 Bsth-Cat RLLPDDTHIM AVVKANAYGH GDVQVARTAL ERGPPP.AVA FLDEALALRE KGIEA  79
    Bsubt KHIGEHVHLM AVEKANAYGH GDAETAKAAL DAGASCLAMA ILDEAISLRK KGLKA  82
  Mleprae EH.AGMAQLM VVLKADAYGH GATQVALAAL AAGAAELGVA TVDEALALRA DGISA  85
      Mtb EH.AGHAQLM AVVKADGYGH GATRVAQTAL GAGAAELGVA TVDEALALRA DGITA 107
   Msmegm EL.AGSADVM AVVKADAYGH GALPVARTAL AAGAAALGVA TIPEALALRE GGITA  87
Ecoli-Cat QA.ATHARVW SVVKANAYGH G.IERIWSAI GATDG.FALL NLEEAITLRE RGWKG  74
Styph-Cat RA.APEARVW SVVKANAYGH G.IERVWSAL GATDG.FAML NLEEAITLRE RGWKG  74
Styph-Bio EL.APASKLV AVVKANAYGH GLLETARTL. PDADA.FGVA RLEEALRLRA GGITQ  73
Ecoli-Bio EX.APASKMV AVVKANAYGH GLLETARTL. PDADA.FXVA RLEEALRLRA GGITK  73
 Hinf-Bio QK.APNSKII AVVKANAYGH GVVFVASTLE QNVDC.FGVA RLEEALALRS NGITK  76
Consensus ---A------ AVVKANAYGH G----A-TAL -A------VA -L-EAL-LR- -GI-- 110

 Bsth-Cat PILVLGA... SRPADAALAA QQRIALTVFR SDWLEE.ASA LYSGPFPIHF HLKMD 130
    Bsubt PILVLGA... VPPEYVAIAA EYDVTLTGYS VEWLQE.A.A RHTKKGSLHF HLKVD 132
  Mleprae PVL...AWLH PPGIDFGPAL LADVQIAVSS VRQLDELLDA VRRTGRTATV TVKAD 137
      Mtb PVL...AWLH PPGIDFGPAL LADVQVAVSS LRQLDELLHA VRRTGRTATV TVKVD 159
   Msmegm PVL...AWLH PPGTDFAPAI AADVEVAVSS RRQLEQVTAA AAEVGRTATV TVKVD 139
Ecoli-Cat PILMLEGFFH AQ..DLEIYD QHRLTTCVHS NWQLKALQNA ..RLKAPLDI YLKVN 125
Styph-Cat PILMLEGFFH AQ..DLEAYD TYRLTTCIHS NWQLKALQNA ..RLNAPLDI YVKVN 125
Styph-Bio PILLLEGFFD AA..DLPTIS AQCLHTAVHN QEQLAALEAV ..ELAEPVTV WMKLD 124
Ecoli-Bio PVLLLEGFFD AR..DLPTIS AQHFHTAVHN EEQLAALEEA ..SLDEPVTV WMKLD 124
 Hinf-Bio PILLLEGFFN EQ..DLPILA VNNIETVVHN HEQLDALKRA ..NLPSPIKV WLKID 127
Consensus PIL-L----- ----D----- -------V-S --QL--L--A ------P--V --K-D 165

 Bsth-Cat TGMGRLGVKD EEETKRIVAL IERHPHFVLE ..GLYTHFAT ADEVNTDYFS YQYTR 183
    Bsubt TGMNRLGVKT EEEVQNVMAI LDRNPRLKCK ..GVFTHFAT ADEKERGYFL MQFER 185
  Mleprae TGLNRNGVVT DQYPAMLTAL QRAVVEDAVR LRGLMSHLVY ADQPDNPSND VQGKR 192
      Mtb TGLNRNGVGP AQFPAMLTAL RQAMAEDAVR LRGLMSHMVY ADKPDDSIND VQAQR 214
   Msmegm TGLSRNGVGA ADYPEVLDVL RRAQADGAIR VRGLMSHLVH GDDPENPFNG LQGQR 194
Ecoli-Cat SGMNRLGFQP DRVLTVWQQL RA..MANV.G EMTLMSHFAE AEHPDG..IS GRMAR 175
Styph-Cat SGMNRLGFQP ERAQTVWQQL RA..MRNV.G EMTLMSHFAQ ADHPEG..IG EAMRR 175
Styph-Bio TGMHRLGVRP EEAEAFYQRL TH..CKNVRQ PVNIVSHFAR ADEPECGATE HQLDI 177
Ecoli-Bio TGMHRLGVRP EQAEAFYHRL TQ..CKNVRQ PVNIVSHFAR ADEPKCGATE KQLAI 177
 Hinf-Bio TGMHRLGVAL DEVDYFYQEL KK..LPQIQP HLGMVSHFSR ADELESDYTQ LQINR 180
Consensus TGM-RLGV-- ---------L ---------- --GL-SHFA- AD-P------ -Q--R 220

 Bsth-Cat FLHMLEWLPS RPP...LVHC ANSAASLRFP DRTFNMVRFG IAMYGLAPSP GIKPL 235
    Bsubt FKELIAPLPL KNL...MVHC ANSAAGLRLK KGFFNAVRFG IGMYGLRPSA DMSDE 237
  Mleprae FAALLAQAHE QGLRFEVAHL SNSSATLSRP DLAFDLVRPG IAVYGLSPVP SR... 244
      Mtb FTAFLAQARE QGVRFEVAHL SNSSATMARP DLTFDLVRPG IAVYGLSPVP AL... 266
   Msmegm LADMRVYARE HGVDYEVAHL CNSPAAMTRP DLAFEMVRPG ISLYGLSPIP ER... 246
Ecoli-Cat IEQAAEGLEC R......RSL SNSAATLWHP EAHFDWVRPG IILYGRSPSG QWRDI 224
Styph-Cat IALATEGLQC A......YSL SNSAATLWHP QAHYDWVRPG IILYGASPSG QWRDI 224
Styph-Bio FNAFCQGKPG Q......RSI AASGGILLWP QSHFDWARPG IILYGVSPLE HKPWG 226
Ecoli-Bio FNTFCEGKPG Q......RSI AASGGILLWP QSHFDWVRPG IILYGVSPLE DRSTG 226
 Hinf-Bio FLSVTKDKQG E......RTI AASGGILFWP KSHFECIRPG IIMYGISPTD ..TIG 227
Consensus F--------- ---------- -NS-A-L--P ---FD-VRPG I--YG-SP-- ----- 275

 Bsth-Cat LPYPLKEAFS LHSRLVHVKK LQPGEKVSYG ATYTAQTKEW IGTIPIGYAD G.VRR 289
    Bsubt IPFQLRPAFT LHSTLSHVKL IRKGESVSYG AEYTAEKDTW IGTVPVGYAD GWLRK 292
  Mleprae GDMGLIPAMT VKCAVAMVKS IRAGEGVSYG HDWIAQHDTN LALLPVGYAD GVFRS 299
      Mtb GDMGLVPAMT VKCAVALVKS IRAGEGVSYG HTWIAPRDTN LALLPIGYAD GVFRS 321
   Msmegm GDMGLRPAMT LKCPVALVRS VHAGDGVSYG HRWVADRDTT LGLLPIGYAD GVYRA 301
Ecoli-Cat ANTGLRPVMT LSSEIIGVQT LKAGERVGYG GRYTARDEQR IGIVAAGYAD GYPRH 279
Styph-Cat ADTGLKPVMT LSSEIIGVQT LSAGERVGYG GGYSVTQEQR IGIVAAGYAD GYPRH 279
Styph-Bio PDFGRQPVMS LTSSLIAVRD HKAGEPVGYG GTWVSERDTR LGVVAMGYGD GYPRA 281
Ecoli-Bio ADFGCQPVMS LTSSLIAVRE HKAGEPVGYG GTWVSERDTR LGVVAMGYGD GY.XA 280
 Hinf-Bio KEFGLTPVMN LTSSLIAVRH HKQGDPVGYG GIWTSPRDTK IGVVAMGYGD GYPRD 282
Consensus -D-GL-P-MT L-S----V-- --AGE-V-YG --W-A--DT- -G-V--GYAD G--R- 330

 Bsth-Cat L.QHFHVLVD GQKAFIVGRI CMDQCMIRL. ..PGPLPVGT KVTLIGRQGD EVISI 340
    Bsubt L.KGTDILVK GKRLKIAGRI CMDQFMVEL. ..DQEYPPGT KVTLIGRQGD EYISM 343
  Mleprae LGGRLDVLIN GKRRPGVGRI CMDQFVVDLG PGPIDVAEGD EAILFGPGAR GEPTA 354
      Mtb LGGRLEVLIN GRRCPGVGRI CMDQFMVDLG PGPLDVAEGD EAILFGPGIR GEPTA 376
   Msmegm LSGRIDVLIK GRRRRAVGRI CMDQFVVDLG PDADDVAVGD DAILFGPGAN GEPTA 356
Ecoli-Cat ALTGTPVLVD GVRTMTVGTV SMDMLAVDLT PCPQAGI.GT PVELWGKEIK IDDV. 332
Styph-Cat APTGTPVLVD GIRTRTVGTV SMDMLAVDLT PCPQAGI.GT PVELWGKEIK VDDV. 332
Styph-Bio APSGTPVLVN GREVPIVGRV AMDMICVDLG PNAQDNA.GD PVVLWGEGLP VERI. 334
Ecoli-Bio APSGTPVLVN GREVPIVGRV AMDMICVDLG PQAQDKA.GD PVILWGEGLP VERI. 333
 Hinf-Bio VPEGTPVYLN GRLVPIVGRV SMDMLTVDLG ADSQDLV.GD EVILWGKELP IETV. 335
Consensus ---GT-VLV- G-R-P-VGR- -MD---VDLG P--QD---GD -V-L-G---- -E--- 385

 Bsth-Cat DDVARHLETI NYEVPCTISY RVPRIFFRHK RIMEVRNAIG RGESSA*386
    Bsubt DEIAGRLETI NYEVACTISS RVPRMFLENG SIMEVRNPLL QVNISN*389
  Mleprae QDWADLLGTI HYEVVTSLRG RITRTYREAQ TVDR*..... .......388
      Mtb QDWADLVGTI HYEVVTSPRG RITRTYREAE NR*....... .......408
   Msmegm QDWAELLDTI HYEVVTSPRG RVTRTYLPAG QQD*...... .......389
Ecoli-Cat ...AAAAGTV GYELMCALAV RVPVVTV*.. .......... .......356
Styph-Cat ...ASAAGTL GYELLCAVAP RVPFVTT*.. .......... .......356
Styph-Bio ...AEMTKVS AYELITRLTS RVAMKYID*. .......... .......359
Ecoli-Bio ...AEMTKVS AYELITRLTS RVAMKYVD*. .......... .......358
 Hinf-Bio ...AKFTGIL SYELITKLTP RVITEYVD*. .......... .......360
Consensus ---A-----T- -YE--T---- RV---Y---- ---------- -------432
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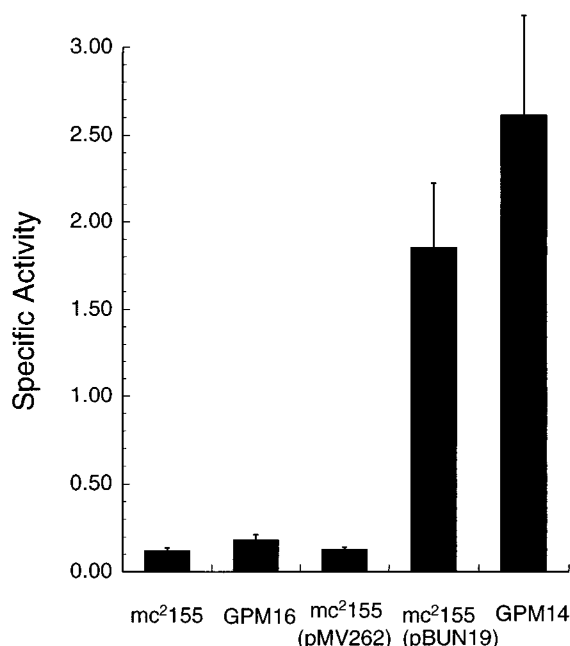

FIG. 5. Analysis of *M. smegmatis* D-alanine racemase activities. Enzyme activity was determined in cell extracts from cells grown in minimal medium (55) without DCS. Specific activities are expressed as micromoles of L-alanine per minute per milligram (means ± standard deviations of triplicate measurements).

box at position −13 in the short transcript. Quantification of radioactivity in the primer extension bands demonstrated that both the shorter and longer transcripts of GPM14 (DCS^r) were overproduced approximately 15-fold with respect to those in mc^2155 (DCS^s).

To assess whether the point mutation in the promoter region of the D-alanine racemase gene of mutant strain GPM14 was responsible for changes in the promoter strength, transcriptional fusions to a reporter gene were performed. The *Sca*I-*Cla*I fragments (Fig. 2), which included approximately 500 bp of the noncoding region of the D-alanine racemase gene from wild-type (mc^2155) and mutant (GPM14) strains, were subcloned into the promoter-probe vector pCV77 (Table 1) upstream from the promoterless *lacZ* gene. These promoter constructs were transformed into wild-type *M. smegmatis* mc^2155, and β-galactosidase activities were measured. The expression from the construct containing the GPM14 *alrA* promoter was approximately 50-fold higher than the expression from the respective mc^2155 promoter. These results (Table 2) confirmed that a point mutation, which increases the %AT of the putative −10 box of the *alrA* gene, led to a significant increase in the level of gene expression.

Overexpression of the *M. smegmatis* D-alanine racemase (*alrA*) gene from a multicopy plasmid in *M. intracellulare* and *M. bovis* BCG leads to a DCS-resistant phenotype. To determine if the overproduction of D-alanine racemase could also confer a DCS^r phenotype in pathogenic mycobacteria, recombinant plasmid pBUN19 was electrotransformed into *M. intracellulare* mc^276 and *M. bovis* BCG. The susceptibilities of the wild-type and transformant strains to DCS are depicted in Fig. 7. These mycobacterial species are inherently more susceptible to DCS than *M. smegmatis*. As illustrated, the *M. intracellulare* strain mc^276(pBUN19) is more resistant to DCS than the parent strain transformed with plasmid vector pMV262 ($P < 0.01$). Colony morphology changes were noticed at DCS concentrations higher than 40 μg ml^{-1} in *M. intracellulare*. A similar profile was obtained with *M. bovis* BCG, where the transformant with plasmid pBUN19 was more resistant than the control strain carrying cloning vector pMV262 ($P < 0.05$). Taken together, these data indicate that multiple copies of a wild-type D-alanine racemase gene can confer a DCS-resistant phenotype in *M. intracellulare* and *M. bovis* BCG.

DISCUSSION

In this study, we identified a gene from *M. smegmatis* that confers a DCS-resistant phenotype to a wild-type DCS-sensitive host when cloned into a multicopy vector. This represents the first molecular genetic analysis of DCS targets in mycobacteria. The mechanisms of DCS resistance in mycobacteria have not been thoroughly investigated. Since DCS can inhibit several pyridoxal phosphate enzymes (31), the existence of more than one mechanism leading to DCS resistance is likely. In addition, multiple-step DCS^r mutants of mycobacteria were isolated in previous studies (46) as well as in our laboratory. The mechanisms relevant to DCS resistance in mycobacteria are presented in the model depicted in Fig. 8. DCS may enter the mycobacterial cell by either diffusion or uptake via a specific transporter (48). Mutations in the transporter gene reducing DCS binding or eliminating the transporter from the cell surface may lead to reduced DCS uptake and increased DCS resistance. Similarly, a mutation in a gene coding for an efflux pump may lead to higher affinity for DCS, with the concomitant expulsion of the drug. Alternatively, it is possible that a mutational change could increase the affinity or the levels of a drug-detoxifying enzyme which would derivatize or hydrolyze DCS. The overproduction of a protein target(s) could lead to increased resistance due to the sequestration or removal of the

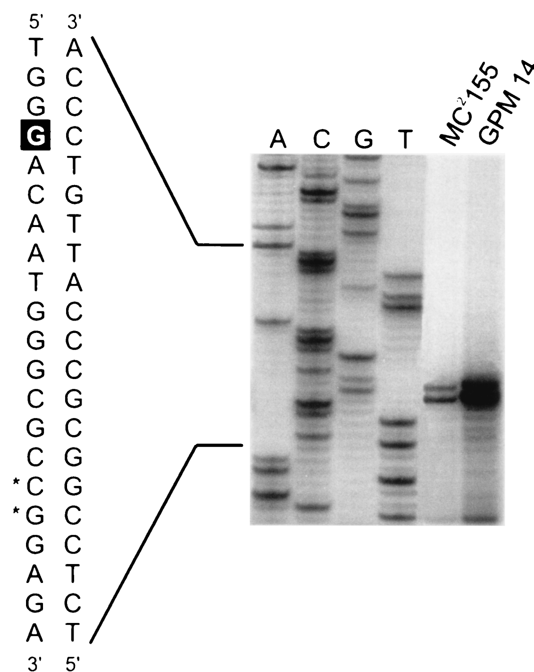

FIG. 6. Primer extension analysis of the *alrA* transcript. Total RNA (50 μg) from mc^2155 or GPM14 cells was annealed to an oligonucleotide of the *alrA* gene and extended as described in Materials and Methods. Lanes A, C, G, and T display a dideoxy sequencing ladder of the wild-type *alrA* gene generated with the same oligonucleotide primer. Nucleotides at the two start sites are indicated by asterisks. The target site for the mutation in the promoter region is boxed.

TABLE 2. Expression of β-galactosidase activity in recombinant *M. smegmatis* carrying transcriptional fusions to *alrA* upstream sequences

Source of *alrA* upstream sequence	Plasmid	Relevant *alrA* upstream sequence[a]	Sp act of β-galactosidase[b]
None	pCV77	None	0.42 ± 0.01
mc^{2}155	pBUN101	GG**G**ACAATGGGCGCC**G**GAGATTATGACGATG	7.92 ± 0.01
GPM14	pBUN102B	GGTACAATGGGCGCC**G**GAGATTATGACGATG	(3.6 ± 0.2) × 10^2

[a] The putative −10 box, Shine-Dalgarno sequence, and ATG start codon for the *alrA* gene are underlined; the T-for-G transversion in the upstream noncoding sequences and the transcriptional start site are shown in boldface type. The transversion increases the similarity of the putative −10 box with the consensus *E. coli* Eσ^{70} promoter (37) and the general consensus established for *M. smegmatis* and *M. tuberculosis* promoters (4).

[b] The specific activities of β-galactosidase from *M. smegmatis* mc^{2}155 transformants carrying promoter constructs are expressed in units/milligram of protein (means ± standard deviations of triplicate measurements).

drug by the excess target protein. The latter mechanism would effectively reduce the free intracellular DCS concentration, which would also protect other potential protein targets from drug inhibition. If DCS interacts with more than one target protein (such as D-alanine racemase and D-alanine ligase), it is unlikely that mutations in one of the structural genes alone would lead to primary DCS resistance. In this study, we presented evidence that one of the mechanisms of DCS resistance in *M. smegmatis* involves the overexpression of the *alrA* gene due to a promoter-up mutation. In agreement with our findings, a similar mechanism of DCS^r involving the overproduction of either D-alanine racemase or D-alanine ligase enzyme activities, possibly mediated by wild-type products, was described for streptococci (38).

We have shown that D-alanine racemase is a target of DCS and that a natural mechanism of resistance is the overproduction of the enzyme due to a promoter-up mutation. An interesting finding was that the original DCS^r mutant, GPM16, used as a source of DNA for the cloning experiments did not possess elevated levels of D-alanine racemase. Hence, the resistance mechanism in GPM16 and three other mutants is not related to the D-alanine racemase determinant. We are currently investigating another DCS^r clone carrying a different DCS resistance determinant (6).

Soon after the introduction of DCS as an antituberculosis drug about 40 years ago, DCS^r clinical isolates of *M. tuberculosis* were readily isolated (8). Since those first trials, DCS has not been administered frequently or alone due to its toxicity in patients. As a consequence, the current panel of DCS^r clinical isolates of pathogenic mycobacteria is limited and may not be representative of the situation that would arise in therapy with DCS. Once we identified a DCS^r determinant in *M. smegmatis* and showed its conservation within the genus, we tested whether a similar mechanism of DCS resistance could occur in members of the pathogenic mycobacterial groups by transforming *M. intracellulare* and *M. bovis* BCG with a multicopy plasmid carrying the *M. smegmatis alrA* gene. DCS^r transformants were obtained, suggesting that AlrA overproduction is a potential mechanism of DCS resistance in clinical isolates of *M. tuberculosis* and other pathogenic mycobacteria. Furthermore, the homology between *M. smegmatis* AlrA and the *M. leprae* and *M. tuberculosis* counterparts suggests a potential utility of *M. smegmatis* as a surrogate host for the study of the AlrA enzymes from pathogenic species.

Since racemases are absent from mammalian cells, these bacterial enzymes are excellent targets for antibiotic development (47). Several compounds were developed but were never advanced into clinical use due to their toxicity in humans (32).

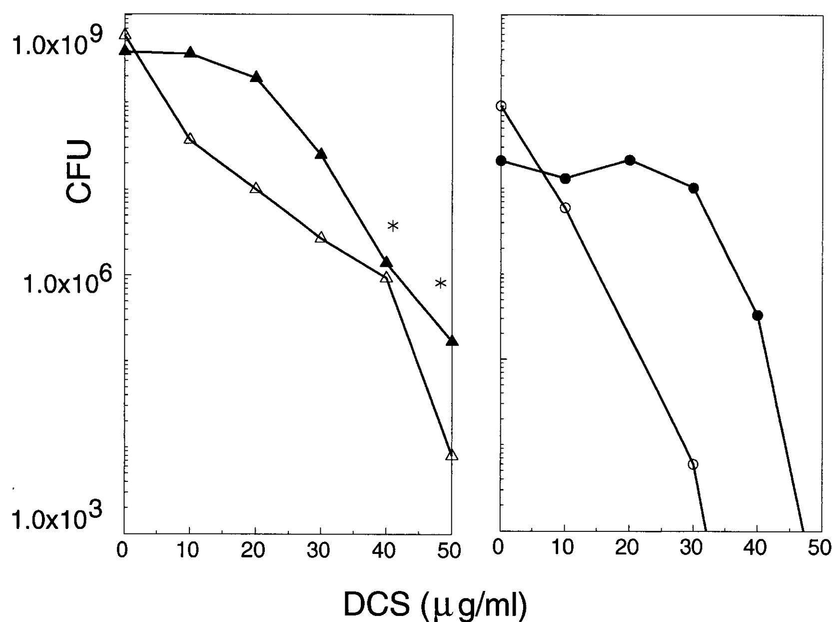

FIG. 7. Inhibition of CFU at increasing DCS concentrations. (Left) Comparison of *M. intracellulare* strains mc^{2}76(pMV262) (open triangles) and mc^{2}76(pBUN19) (closed triangles). (Right) Comparison of *M. bovis* BCG(pMV262) (open circles) and BCG(pBUN19) (closed circles). The asterisks indicate the concentrations at which changes in colony morphology (from opaque white domed to transparent flat colonies) were observed. For analysis of statistical significance, see the text.

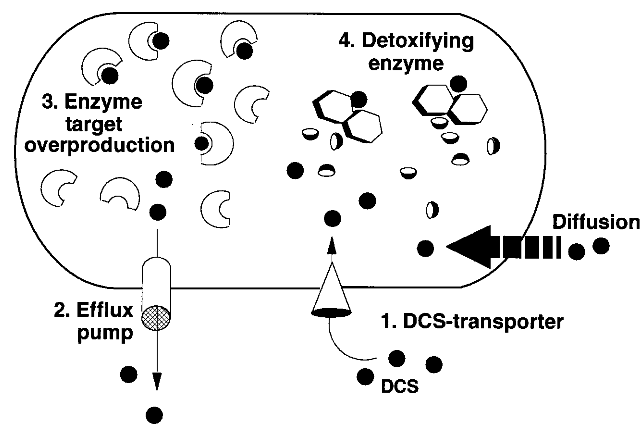

FIG. 8. Model of DCS resistance mechanisms in mycobacteria. There are four proposed mechanisms of resistance: impairments in the DCS transporter, acquisition or overproduction of an efflux pump, enzyme target overproduction, and acquisition or overproduction of a DCS-detoxifying enzyme.

The D-alanine racemase gene of mycobacteria may be a powerful tool for the rational design of effective and less toxic DCS derivatives and D-alanine analogs against *M. tuberculosis* and other pathogenic mycobacteria. A structure-based layout in which computational and crystallography methods are combined would advance the rational design of a new generation of drugs. A key ingredient to the success of this strategy is the identification of the DCS lethal target(s) in pathogenic mycobacteria whose inhibition leads to cell death.

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