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## Potential Drug Targets for *Mycobacterium avium* Defined by Radiometric Drug-Inhibitor Combination Techniques

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Previously established radiometric techniques were used to assess the effectiveness of combined antimicrobial drug-inhibitory drug (drug-inhibitor) treatment on two clinical isolates of the *Mycobacterium avium* complex representing three colony variants: smooth opaque (dome) (SmO), smooth transparent (SmT), and rough (Rg). All variants were identified as members of the *M. avium* complex; however, only the SmT colony type of strain 373 possessed characteristic serovar-specific glycopeptidolipid (GPL) antigens. MICs, determined radiometrically, of drugs with the potential to inhibit the biosynthesis of GPL antigens or other cell envelope constituents were similar for all strains. These drugs included cerulenin, *N*-carbamyl-DL-phenylalanine, *N*-carbamyl-L-isoleucine, *trans*-cinnamic acid, ethambutol, 1-fluoro-1-deoxy- $\beta$ -D-glucose, 2-deoxy-D-glucose, and *m*-fluoro-phenylalanine. The MICs of the antimicrobial drugs amikacin, sparfloxacin, and clarithromycin varied, but overall the MICs for the SmO variant were the lowest. Radiometric assessment of drug-inhibitor combinations by using established *x/y* determinations revealed enhanced activity when either ethambutol or cerulenin were used in combination with all antimicrobial agents for all variants except the Rg variant of strain 424, for which ethambutol was not effective. Enhanced activity with amino acid analogs was observed with the Rg colony variants of strains 373 and 424. Two potential sites for drug targeting were identified: fatty acid synthesis, for all strains assayed, and peptide biosynthesis, particularly for Rg colony variants that possess previously identified phenylalanine-containing lipopeptides as potential targets for future drug development.

Treatment of infections caused by members of the *Mycobacterium avium* complex is difficult because of an intrinsic resistance that these organisms have to most of the antimicrobial agents generally used to treat other mycobacterial infections (15, 22, 33). Moreover, monotherapy of *M. avium* infections with clarithromycin in clinical trials is clearly associated with an increase in MICs and relapses (12). For example, initial MICs of as low as  $\leq 2.0$   $\mu\text{g/ml}$  before treatment increased to  $\geq 256$   $\mu\text{g/ml}$  at the time of relapse (18). It is therefore apparent that better control of *M. avium* disease will require multiple-drug therapy with appropriate new drug combinations, particularly because the present drug regimens with commercially available drugs have not shown appreciable efficacy in eliminating infections caused by members of the *M. avium* complex (15, 17).

The most likely explanation for the diverse drug susceptibility patterns of *M. avium* strains is the cell envelope architecture, which probably acts as an exclusion barrier for some drugs (13, 29). Among the several components present in the *M. avium* cell envelope that may contribute to this drug exclusion barrier are the serovar-specific glycopeptidolipid (GPL) antigens. The GPLs are unique to the *M. avium* complex, and because of their noncovalent linkage in the cell envelope, they deposit themselves around the mycobacteria (4, 37) and accumulate during prolonged growth in macrophages following infection (34). In addition, the GPLs are slowly degraded substances (21, 39) that have immunomodulatory

properties (3, 9, 27) and may play a role in pathogenicity, in addition to their possible contribution to drug resistance.

In an effort to design effective drug therapy for the treatment of *M. avium*, we used a combined drug-inhibitor treatment that would potentially inhibit GPL biosynthesis, thereby affecting cell envelope architecture and thus allowing more effective use of antimicrobial agents that have demonstrated efficacy in *M. avium* therapy. For this strategy, we used drugs that have the potential to affect GPL biosynthesis at three sites: glycosylation, peptide biosynthesis, and fatty acid biosynthesis. We have reported that this approach is capable of producing a synergistic effect when drugs which potentially inhibit GPL biosynthesis are used in combination with amikacin, sparfloxacin, and clarithromycin (5). Those studies were an extension of an earlier report of combined treatment with *m*-fluoro-phenylalanine, an inhibitor GPL biosynthesis (14), and ethambutol, an inhibitor of arabinogalactan biosynthesis (36). That combination was effective in producing synergistic growth inhibition of *M. avium* (32).

In our most recent publication regarding this combined drug-inhibitor treatment we used laboratory-maintained isolates: a smooth opaque (dome) (SmO) and a rough (Rg) variant of *M. avium* serovar 4 (5). In this report we extend our observations to a clinical isolate which presented with the SmO colony morphology upon initial isolation on Lowenstein-Jensen medium but, upon successive subculturing on 7H9 agar, segregated into distinct colony morphotypes: smooth transparent (SmT), SmO, and Rg. All three morphotypes were identified as members of the *M. avium* complex. We also examined the effects of combined drug-inhibitor treatment on another Rg clinical isolate to confirm the difference in susceptibility that we observed for Rg and Sm colony variants. Internal radiolabeling techniques were used to confirm the presence or

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absence of GPL and GPL-related components and to further confirm the presence of a unique phenylalanine-isoleucine-alanine-containing lipopeptide that appears to be common to both Sm and Rg colony variants of *M. avium* and that may also be a factor in the increased susceptibilities of Rg variants to treatment with various drug-inhibitor combinations.

## MATERIALS AND METHODS

**Mycobacteria.** The mycobacterial strains that were used in the investigation were isolated from two separate patients. Upon initial isolation at the Institut Pasteur (Paris, France), strain 373 had a characteristic Sm colony morphology on Lowenstein-Jensen medium and strain 424 had a characteristic Rg colony morphology. Both strains were identified as *M. avium* complex by standard biochemical procedures and Genprobe analysis but were not typeable by thin-layer chromatographic (TLC) techniques (38) because they did not possess the characteristic serotype-specific GPL (C-mycoside) antigens (16). This is not an unusual finding for Rg strains because they lack the characteristic serovar-specific GPL antigens (2, 7). However, because this finding was unusual for the Sm variant, both strains were sent to W. W. Barrow's laboratory for further examination. Subculturing of strain 424 on 7H9 agar plates (prepared by adding agar to Difco's 7H9 broth) revealed that it retained its Rg morphology. Subculturing of strain 373 on 7H9 agar resulted in three distinct colony types representing SmO, SmT, and Rg colony variants. The order of frequency was SmO > SmT > Rg. Independent examination of all four isolates by standard biochemical procedures, Genprobe and mycolic acid patterns (11), confirmed that they were all members of the *M. avium* complex.

**Internal radiolabeling of mycobacterial lipids.** The procedure for internal radiolabeling has been published previously (21, 39); therefore, it will only be given here briefly. Mycobacteria were cultured in 7H9 Middlebrook broth (Difco) containing glycerol and oleic acid-albumin-dextrose. During growth, either L-[U-<sup>14</sup>C]phenylalanine ([<sup>14</sup>C]Phe; 405 mCi/mM) or L-[U-<sup>14</sup>C]isoleucine ([<sup>14</sup>C]Ile; 166 mCi/mM) (ICN Radiochemicals, Inc., Irvine, Calif.) was added at a concentration of 0.05 μCi/ml. In order to obtain optimum dispersal of the Rg variants, it was necessary to add glass beads (3-mm diameter) to the culture flasks (5). Following incorporation of the radiolabel, the mycobacteria were autoclaved and harvested by centrifugation. The cells were then lyophilized and stored at -20°C until the lipids were extracted (5).

**Purification of lipids.** Lipids were extracted from lyophilized mycobacteria by the Folch procedure as described previously (5). Lipids were examined by TLC by first applying 100 μg of lipid and then developing the plate in either chloroform-methanol-water (60:12:1) (solvent A) or chloroform-methanol (11:1) (solvent B). TLC plates were sprayed with an orcinol-sulfuric acid reagent to confirm the presence or absence of GPLs (5, 8).

**HPLC procedures.** Lipids were analyzed by high-performance liquid chromatography (HPLC) by using a Beckman System Gold model 126 programmable solvent delivery module controlled by System Gold chromatography software as described previously (5). Fractions were separated on an Ultrasphere 5-μm Spherical 8.0-nm-pore-size SI analytical column (4.6 by 250 mm) attached to a guard column containing the same solid support (4.6 by 45 mm) and were detected with a 171 solid system radioisotope detector (5). Samples were injected (400 μg) and were separated in a mobile phase of 100% chloroform for 10 min and then a 40-min gradient of 0 to 10% methanol in chloroform at a flow rate of 1.0 ml/min

(41). Data from DIF files were converted into graphic form by use of an Excel (Microsoft) program (10) and printed from a Macintosh PowerBook 140 as described previously (5).

**Growth conditions for inhibitory studies.** Growth of the mycobacteria was monitored radiometrically by means of a BACTEC 460-TB instrument (Becton Dickinson, Towson, Md.) as reported previously (35). Growth was measured as a function of the release of <sup>14</sup>C-labeled CO<sub>2</sub> resulting from the metabolism of <sup>14</sup>C-labeled palmitate in Middlebrook 7H12b broth (35). Growth was then expressed as a numerical value, referred to as the growth index (GI), that ranges from 1 to 999 (35).

Preparation of samples for experimental procedures was conducted as described previously (32), but is described here briefly. A vial containing 4 ml of broth (primary culture) was inoculated with 0.1 ml of mycobacterial suspension (optical density of 0.15 at 650 nm, measured with a Coleman Junior II spectrophotometer). When the primary culture had reached a GI of 500, a portion was removed and diluted 10-fold. Then, 0.1 ml of this diluted portion was injected directly into drug-containing vials.

Interpretation of MICs was accomplished by comparing the GIs of the drug-containing vials with those of their respective controls inoculated with 100-fold fewer bacteria (1:100 control) (19). MICs were interpreted once the GI in the control vial had reached a value of 30 or more, as described previously (19, 31).

The *x/y* quotient calculation method was used to evaluate the combined drug (plus inhibitor) action (20, 32). For this procedure, all of the drugs and inhibitors were used at sublethal concentrations. For the *x/y* quotient determinations, a preculture with a GI of 500 was diluted 10-fold and 0.1 ml of the suspension was injected into drug-containing vials. The combined drug-inhibitor activity was assessed by calculating *x/y* quotients as follows. The *x* value was the GI obtained with the combination of drug-inhibitor by using the BACTEC instrument, whereas the *y* value was the lowest GI obtained at the same time with the drug or the inhibitor used alone. For combinations, an *x/y* value of 1 indicated that there was no interaction between the two drugs, a quotient of <0.5 indicated enhanced drug action, whereas an *x/y* quotient of >2 indicated the presence of antagonism between the drug and the inhibitor.

**Drugs and inhibitors.** The following drugs were used (concentrations are given in the appropriate tables because the MICs varied for each of the strains): amikacin, sparflaxacin, and clarithromycin. In addition, the following inhibitors were used: ethambutol, cerulenin, *N*-carbamyl-DL-phenylalanine, *N*-carbamyl-L-isoleucine, *trans*-cinnamic acid, *m*-fluoro-phenylalanine, and 1-fluoro-1-deoxy-β-D-glucose (Sigma Chemical Co., St. Louis, Mo.). Sparflaxacin (Rhône D.P.C. Europe, Antony, France), amikacin (Bristol, Paris, France), and clarithromycin (Abbott Laboratories, North Chicago, Ill.) were kindly provided by their manufacturers. For stock solutions of clarithromycin, *N*-carbamyl-L-isoleucine, and *m*-fluoro-phenylalanine, methanol was used to dissolve the drugs, and in the case of cerulenin, absolute ethanol was used (5). All other inhibitors and drugs were dissolved in distilled water and the stock solution was sterilized by filtration through a 0.22-μm-pore-size filter before addition to test vials (5). Appropriate controls were included to ensure that the inhibitory effects of the drugs were not due to the addition of solvent.

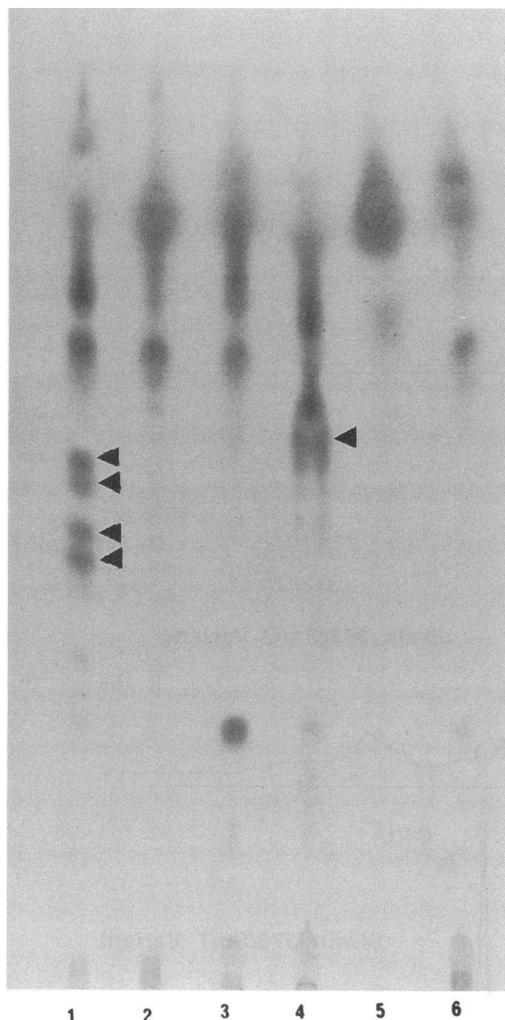


FIG. 1. TLC plate containing lipid extracted from *M. avium* variants of strains 373 and 424. Lipid (100  $\mu$ g each) from SmO, SmT, and Rg variants of strain 373 was applied to lanes 3 through 5, respectively. Lipid (100  $\mu$ g) from Rg variant strain 424 was applied to lane 6, and lipid (100  $\mu$ g each) from *M. avium* serovar 4 SmO and Rg variants (see reference 5) was applied to lanes 1 and 2, respectively. The plate was developed in solvent A, and GPLs (indicated by arrows) were detected with orcinol-sulfuric acid reagent.

## RESULTS

**TLC analysis of lipids from *M. avium* isolates.** Lipids were extracted by the Folch procedure (3) and were examined by TLC by using solvent A, which gives better separation of the more polar GPL (Fig. 1), and solvent B (data not shown), which gives better separation of the apolar GPL. When lipids were developed in solvent A and exposed to orcinol-sulfuric acid reagent (23) along with authentic lipids extracted from a SmO and a Rg colony variant of *M. avium* serotype 4, it was observed that lipid extracted from the SmT variant of strain 373 contained components that had the characteristic yellow-gold color indicative of GPL (Fig. 1). The lipids from the SmO or the Rg variant of strain 373 did not contain any such components. Examination of lipids by using solvent B revealed that lipids from the SmO and SmT variants of strain 373 did contain lipids characteristic of the apolar GPL. No evidence of

GPL or apolar GPL was observed in the lipid pattern from the Rg variant of either strain (Figure 1).

**HPLC analysis of radiolabeled lipids.** Individual cultures of each isolate were grown in 7H9 broth in the presence of either [ $^{14}$ C]Phe or [ $^{14}$ C]Ile in order to internally radiolabel the GPL and GPL-related components (5, 21, 39). Radiolabeled lipids were then separated and analyzed by HPLC procedures described previously (5, 41). In Figure 2, characteristic HPLC patterns are shown for the [ $^{14}$ C]Phe-labeled lipids for all four strains. Examination of lipid extracted from the Rg variants of strain 424 (Fig. 2A) and strain 373 (Fig. 2D) revealed a complete absence of GPL antigens and apolar GPL. Only one major peak was observed; that peak had a retention time identical to that of a Phe-Ile-Ala-containing lipopeptide that we described previously in both Sm and Rg variants of *M. avium* (5). This peak was also observed in lipid extracted from the SmO, SmT, and Rg variants of strain 373 (Fig. 2B to D, respectively). Although the relative height for peak I, in the case of the SmO variant, appears to be small in comparison with those for the SmT and Rg variants of the same strain, it should be noted that the peak areas were very similar for all three variants. The areas for peak I for SmO, SmT, and Rg variants were 3,165, 3,176, and 3,417 cpm, respectively. One of the reasons for this difference in [ $^{14}$ C]Phe incorporation into peak I was the presence of other phenylalanine-containing lipopeptides (retention time, 25 to 27 min) that had incorporated most of the radiolabel (Fig. 2B). Because of this, the amount of peak I appeared to be reduced in the case of the SmO variant. Although not further characterized, these phenylalanine-containing lipopeptides have retention times similar to those of phenylalanine-containing lipopeptides reported previously in *M. avium* serovar 4 (5, 40) and serovars 8 and 20 (40) and may play a role in GPL biosynthesis (5). The area for peak I of the Rg strain 424 was 47,828 cpm; however, that strain grew faster and produced a better cell yield than did the three variants of strain 373.

Peaks corresponding to GPL were observed in lipid from SmT (Fig. 2C). The locations of GPLs in HPLC fractions were confirmed by analysis on TLC plates by using the orcinol-sulfuric reagent (23). Confirmation of radiolabel incorporation was accomplished as described previously by hydrolysis of appropriate HPLC fractions in HCl and analysis by means of cellulose TLC by using authentic amino acid standards and scintillation counting of the corresponding sections on cellulose TLC plates (21, 41).

To confirm the location of the Phe-Ile-Ala-containing lipopeptide, additional lipid fractions, obtained from cells exposed to [ $^{14}$ C]Ile, were analyzed by HPLC. Figure 3 represents a typical HPLC pattern of the [ $^{14}$ C]Ile-labeled lipids obtained from all four variants. Only a single peak was observed for all of the variants. This single [ $^{14}$ C]Ile-labeled component (Fig. 3) had a retention time identical to that observed for peak I in the [ $^{14}$ C]Phe-labeled lipids (Fig. 2) and, in addition, cochromatographed with the previously identified phenylalanine-isoleucine-alanine-containing lipopeptide reported earlier (HPLC pattern not shown) (1, 5).

**Radiometric determination of inhibitory drug effects on *M. avium* growth.** Inhibitory effects were monitored and the MICs of each individual inhibitory drug were determined. No inhibition was observed for *N*-carbonyl-L-isoleucine, *N*-carbonyl-DL-phenylalanine, 1-fluoro-1-deoxy-D-glucose, or 2-deoxy-D-glucose (Table 1). The most effective drug was cerulenin, which demonstrated low MICs for all four strains (Table 1). *m*-Fluoro-phenylalanine was effective for all strains, as was *trans*-cinnamic acid, with only slight differences observed between strains (Table 1). The MICs obtained for the antimicro-

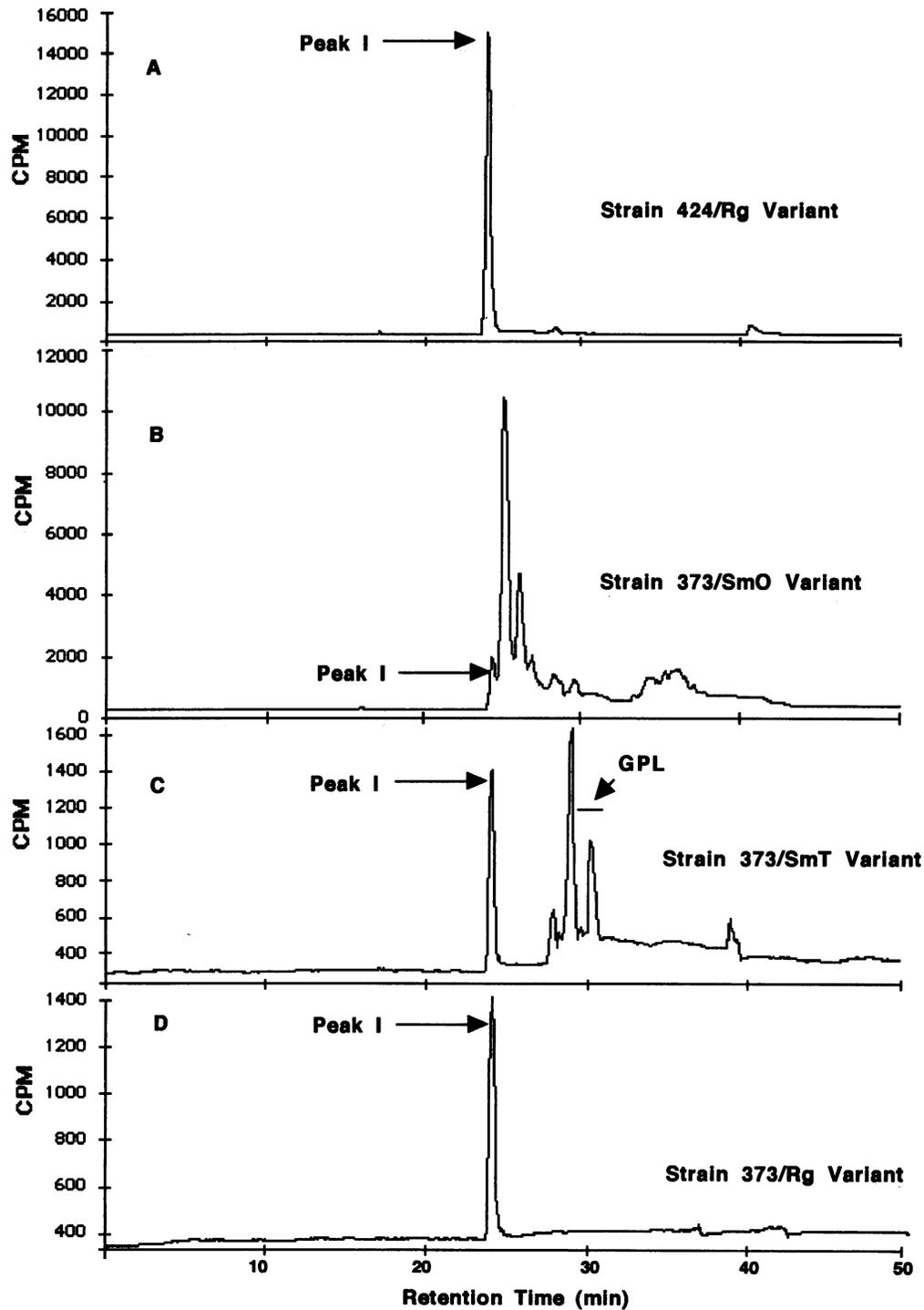


FIG. 2. HPLC separation of [ $^{14}\text{C}$ ]Phe-labeled native lipid extracted from Rg variant strain 424 (A), SmO variant strain 373 (B), SmT variant strain 373 (C), and Rg variant strain 373 (D). Peak I as well as GPL are indicated with arrows. Column conditions are given in the text.

bial agents varied, with the SmT and Rg variants of strain 373 being more resistant to sparflaxacin (Table 1). The one exception was with ethambutol, whose MIC was the same for all three variants (Table 1).

**Radiometric determinations for combined drug treatment.** By using the  $x/y$  quotient calculations established previously (32), the results obtained from combined drug-inhibitor action

for all of the variants of *M. avium* were assessed and are given in Tables 2 through 5. In addition, representative radiometric data for some of the combinations and parallel controls are given in Fig. 4A through C. For all three colony types of strain 373, an enhanced drug-inhibitor activity was observed for combinations that included ethambutol or cerulenin (Tables 2 through 4 and Fig. 4A and B). Only one representative graph

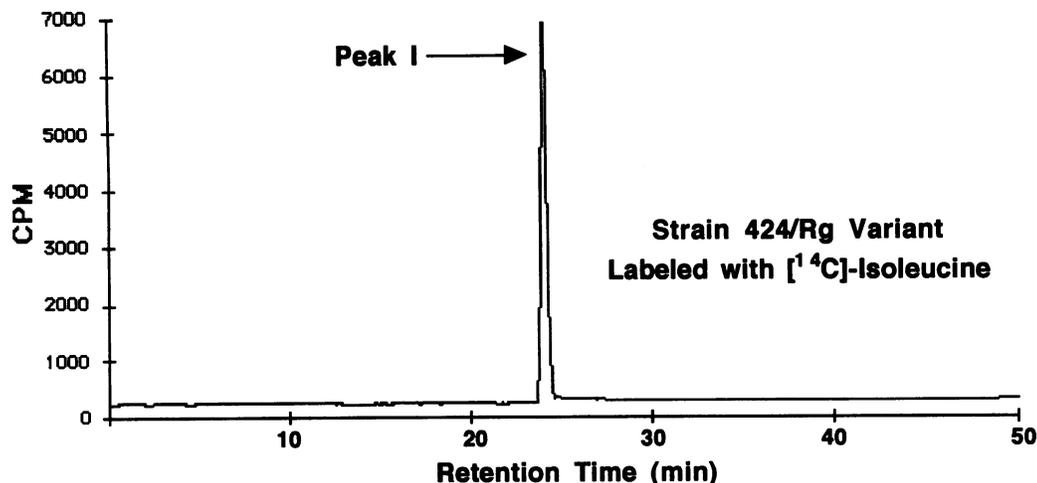


FIG. 3. HPLC pattern of [ $^{14}\text{C}$ ]Ile-labeled native lipid extracted from Rg variant strain 424. This pattern is identical to those obtained from [ $^{14}\text{C}$ ]Ile-labeled lipids extracted from SmO, SmT, and Rg variants of strain 373. Peak I is indicated with an arrow. Column conditions are given in the text.

is given for the enhanced activity of ethambutol or cerulenin plus an antimicrobial agent (i.e., amikacin, sparfloxacin, and clarithromycin) in the case of strain 373 SmO and SmT (Fig. 4A) because the results were very similar. Graphic results for the other drug-inhibitor combinations are not given because there was no enhanced activity for the SmO or SmT variants of strain 373. No enhanced activity was observed for combinations with ethambutol in the case of the Rg variant of strain 424, even though the ethambutol MIC for that strain was equivalent to those for the variants of strain 373 (Tables 1 and 5). For the Rg colony variant of strain 373 (Tables 2 through 4 and Fig. 4B) and the Rg variant of strain 424 (Table 5 and Fig. 4C), an enhanced drug-inhibitor activity was additionally observed for antimicrobial combinations that included either *N*-carbamyl-phenylalanine, *N*-carbamyl-isoleucine, *trans*-cinnamic acid, or *m*-fluoro-phenylalanine. No enhanced activity was observed for any of the strains when antimicrobial combinations included 2-deoxy-D-glucose (data not shown), and

enhanced activity with 1-fluoro-1-deoxy-D-glucose was observed only in the case of combined treatment with clarithromycin for the SmT colony variant of strain 373 (Table 4).

## DISCUSSION

Because of its diversified drug resistance patterns to most antimycobacterial agents, the *M. avium* complex presents a major problem in the clinical management of human immunodeficiency virus-infected patients. It is apparent that to achieve successful treatment of *M. avium* infections it will be necessary to use appropriate drug combinations. Although antimicrobial combinations have been used with some success, there remains a need for improved drug therapy for these drug-resistant opportunistic pathogens. Newer types of treatment should be examined to accomplish the necessary improvement in therapy that is required, particularly in the case

TABLE 1. Radiometric MICs of various antimicrobial agents and inhibitory drugs for colony variants of *M. avium* strains

Drug	MIC ( $\mu\text{g/ml}$ )			
	Strain 373			Strain 424 Rg variant
	SmO variant	SmT variant	Rg variant	
Cerulenin	1	5	5	10
NC-Phe	>500	>500	>500	>500
NC-Ile	>500	>500	>500	>500
Cinnamic acid	50	50	50	500
2-DDG	>5,000	>5,000	>5,000	>5,000
Fl-DG	>500	>500	>500	>500
Fl-Phe	200	200	200	200
EMB	5	5	5	5
AMIK	1	2	2	4
SPAR	8	16	16	8
CLAR	<1	2	1	2

<sup>a</sup> Abbreviations: NC-Phe, *N*-carbamyl-DL-phenylalanine; NC-Ile, *N*-carbamyl-L-isoleucine; 2-DDG, 2-deoxy-D-glucose; Fl-DG, 1-fluoro-1-deoxy-D-glucose; Fl-Phe, *m*-fluoro-phenylalanine; EMB, ethambutol; AMIK, amikacin; SPAR, sparfloxacin; CLAR, clarithromycin.

TABLE 2. Combined drug-inhibitor activity against *M. avium* 373 (SmO, SmT, and Rg variants) assessed by radiometric *x/y* quotient determination

Inhibitory drug (concn [ $\mu\text{g/ml}$ ]) <sup>a</sup>	Enhanced activity for amikacin ( <i>x/y</i> quotient) <sup>b</sup>		
	SmO variant (0.25) <sup>c</sup>	SmT variant (0.5) <sup>c</sup>	Rg variant (0.5) <sup>c</sup>
EMB (0.5)	++ (0.1)	++ (0.08)	+++ (0.02)
Cerulenin (0.5)	+++ (0.03)	+ (0.11)	+++ (0.04)
NC-Phe (50)	—	—	—
NC-Ile (100)	—	—	—
Cinnamic acid (10)	—	—	+ (0.47)
Fl-DG (100)	—	—	—
Fl-Phe (50)	—	—	± (0.5)

<sup>a</sup> Abbreviations: EMB, ethambutol; NC-Phe, *N*-carbamyl-DL-phenylalanine; NC-Ile, *N*-carbamyl-L-isoleucine; Fl-DG, 1-fluoro-1-deoxy-D-glucose; Fl-Phe, *m*-fluoro-phenylalanine.

<sup>b</sup> Values in parentheses in the table body are given as the quotient of *x/y*, where *x* is the Bactec GI obtained with the drug-inhibitor combination and *y* is the minimal GI value for the drug or the inhibitor used alone. An *x/y* of <0.5 indicates enhanced drug activity. For two-drug combinations, *x/y* values of >0.5 (—),  $\leq 0.5$  (+),  $\leq 0.1$  (++) ,  $\leq 0.05$  (+++), and  $\leq 0.01$  (++++) are given.

<sup>c</sup> Values in parentheses are the subinhibitory concentrations (in micrograms per milliliter) used for *x/y* quotient determinations.

TABLE 3. Combined drug-inhibitor activity against *M. avium* 373 (SmO, SmT, and Rg variants) assessed by radiometric *x/y* quotient determination

Inhibitory drug (concn [ $\mu\text{g/ml}$ ]) <sup>a</sup>	Enhanced activity for sparfloxacin ( <i>x/y</i> quotient) <sup>b</sup>		
	SmO variant (2) <sup>c</sup>	SmT variant (4) <sup>c</sup>	Rg variant (4) <sup>c</sup>
EMB (0.5)	+ (0.12)	++ (0.1)	+++ (0.016)
Cerulenin (0.5)	+++ (0.02)	+ (0.15)	++ (0.076)
NC-Phe (50)	—	—	+ (0.41)
NC-Ile (100)	—	—	+ (0.29)
Cinnamic acid (10)	—	—	+ (0.25)
Fl-DG (100)	—	—	—
Fl-Phe (50)	—	—	+ (0.43)

<sup>a</sup> Abbreviations: EMB, ethambutol; NC-Phe, *N*-carbonyl-DL-phenylalanine; NC-Ile, *N*-carbonyl-L-isoleucine; Fl-DG, 1-fluoro-1-deoxy-D-glucose; Fl-Phe, *m*-fluoro-phenylalanine.

<sup>b</sup> Values in parentheses in the table body are given as the quotient of *x/y*, where *x* is the Bactec GI obtained with the drug-inhibitor combination and *y* is the minimal GI value for the drug or the inhibitor used alone. An *x/y* of <0.5 indicates enhanced drug activity. For two-drug combinations, *x/y* values of >0.5 (—),  $\leq 0.5$  (+),  $\leq 0.1$  (++) ,  $\leq 0.05$  (+++) , and  $\leq 0.01$  (++++) are given.

<sup>c</sup> Values in parentheses are the subinhibitory concentrations (in micrograms per milliliter) used for *x/y* quotient determinations.

of immunocompromised patients, such as those coinfecting with the human immunodeficiency virus.

Previously, we have used such reasoning in developing drug-inhibitor combinations to achieve increased activity for antimicrobial drugs that have shown some success in the therapy of *M. avium*. This reasoning has been based on the premise that the cell envelope of *M. avium* is probably responsible for the diverse resistance that this organism demonstrates to various antimicrobial agents. We have specifically chosen inhibitors that have the potential to inhibit cell envelope components such as the GPL, in anticipation that this action would help to weaken the cell envelope and augment the inhibitory effects of antimicrobial agents that require entry into the cell for optimum activity. This reasoning appeared to be justified when we demonstrated that combined drug-inhibitor combinations did achieve increased levels of activity when

TABLE 4. Combined drug-inhibitor activity against *M. avium* 373 (SmO, SmT, and Rg variants) assessed by radiometric *x/y* quotient determination

Inhibitory drug (concn [ $\mu\text{g/ml}$ ]) <sup>a</sup>	Enhanced activity for clarithromycin ( <i>x/y</i> quotient) <sup>b</sup>		
	SmO variant (0.1) <sup>c</sup>	SmT variant (0.5) <sup>c</sup>	Rg variant (0.25) <sup>c</sup>
EMB (0.5)	+ (0.24)	++ (0.08)	+++ (0.02)
Cerulenin (0.5)	+ (0.27)	+++ (0.05)	+ (0.17)
NC-Phe (50)	—	—	+ (0.24)
NC-Ile (100)	—	—	+ (0.28)
Cinnamic acid (10)	—	—	+ (0.24)
Fl-DG (100)	—	+ (0.28)	—
Fl-Phe (50)	—	—	+ (0.27)

<sup>a</sup> Abbreviations: EMB, ethambutol; NC-Phe, *N*-carbonyl-DL-phenylalanine; NC-Ile, *N*-carbonyl-L-isoleucine; Fl-DG, 1-fluoro-1-deoxy-D-glucose; Fl-Phe, *m*-fluoro-phenylalanine.

<sup>b</sup> Values in parentheses in the table body are given as the quotient of *x/y*, where *x* is the Bactec GI obtained with the drug-inhibitor combination and *y* is the minimal GI value for the drug or the inhibitor used alone. An *x/y* of <0.5 indicates enhanced drug activity. For two-drug combinations, *x/y* values of >0.5 (—),  $\leq 0.5$  (+),  $\leq 0.1$  (++) ,  $\leq 0.05$  (+++) , and  $\leq 0.01$  (++++) are given.

<sup>c</sup> Values in parentheses are the subinhibitory concentrations (in micrograms per milliliter) used for *x/y* quotient determinations.

TABLE 5. Combined drug-inhibitor activity against *M. avium* 424 (Rg), assessed by radiometric *x/y* quotient determination

Inhibitory drug (concn [ $\mu\text{g/ml}$ ]) <sup>a</sup>	Enhanced activity for antimicrobial agents ( <i>x/y</i> quotient) <sup>b</sup>		
	Amikacin (1.0) <sup>c</sup>	Sparfloxacin (2.0) <sup>c</sup>	Clarithromycin (0.5) <sup>c</sup>
EMB (0.5)	—	—	—
Cerulenin (1.0)	+++ (0.039)	+ (0.19)	+ (0.46)
NC-Phe (50)	+ (0.34)	+ (0.13)	++ (0.07)
NC-Ile (100)	+ (0.36)	+ (0.13)	++ (0.08)
Cinnamic acid (100)	+ (0.21)	++ (0.075)	+ (0.13)
Fl-DG (100)	—	—	—
Fl-Phe (50)	+ (0.35)	+ (0.14)	+ (0.12)

<sup>a</sup> Abbreviations: EMB, ethambutol; NC-Phe, *N*-carbonyl-DL-phenylalanine; NC-Ile, *N*-carbonyl-L-isoleucine; Fl-DG, 1-fluoro-1-deoxy-D-glucose; Fl-Phe, *m*-fluoro-phenylalanine.

<sup>b</sup> Values in parentheses in the table body are given as the quotient of *x/y*, where *x* is the Bactec GI obtained with the drug-inhibitor combination and *y* is the minimal GI value for the drug or the inhibitor used alone. An *x/y* of <0.5 indicates enhanced drug activity. For two-drug combinations, *x/y* values of >0.5 (—),  $\leq 0.5$  (+),  $\leq 0.1$  (++) ,  $\leq 0.05$  (+++) , and  $\leq 0.01$  (++++) are given.

<sup>c</sup> Values in parentheses are subinhibitory concentrations (in micrograms per milliliter) used for *x/y* quotient determinations.

potential inhibitors of GPL biosynthesis were used in combination with amikacin, sparfloxacin, and clarithromycin (5). As a continuation of those studies, we report here on the increased activities of those same antimicrobial agents with additional potential inhibitors of GPL biosynthesis by using clinical isolates that represent the three major colony variants isolated from patients: SmO, SmT, and Rg.

Using a strategy similar to that reported previously (5), we chose drugs that had the potential to inhibit GPL biosynthesis at three sites: glycosylation, peptide biosynthesis, and fatty acid biosynthesis. The two drugs used to inhibit glycosylation were 2-deoxy-D-glucose and 1-fluoro-1-deoxy-D-glucose. These two drugs did not demonstrate low MICs and, with one exception, did not produce increased levels of activity when used in combination with any of the antimicrobial agents. This was not unexpected with the Rg variants, because they lack the ability to synthesize the GPL and would thus not be expected to respond (5). We were surprised to see a lack of activity in the case of the SmT because it appears that that variant does synthesize GPL. Because of our previous findings with 2-deoxy-D-glucose (5), we expected some increased activity. However, the lack of activity with regard to the SmO variant was probably due to the apparent lack of GPL, as evidenced by TLC and HPLC examination.

*N*-Carbonyl-DL-phenylalanine, *N*-carbonyl-L-isoleucine, *trans*-cinnamic acid, and *m*-fluoro-phenylalanine were used to inhibit peptide synthesis. These drugs did show increased levels of activity, but only in the case of the Rg variants. This was expected because similar drugs produced that same effect with a Rg variant of *M. avium* serovar 4 (5). It has been demonstrated that *N*-carbonyl-DL-phenylalanine (14) and *m*-fluoro-phenylalanine (14, 30, 32) inhibit GPL biosynthesis, which probably weakens the cell envelope (30) and allows access of the antimicrobial agents into the cell.

To inhibit fatty acid biosynthesis we again chose cerulenin. Cerulenin has low MICs and, in all cases that we have examined, results in increased drug-inhibitor activities for all three colony variants. Cerulenin has previously been shown to have low MICs for *M. avium* (26), but to our knowledge our studies are the only ones that demonstrate its effectiveness in drug-inhibitor combinations. Because the mycobacterial cell

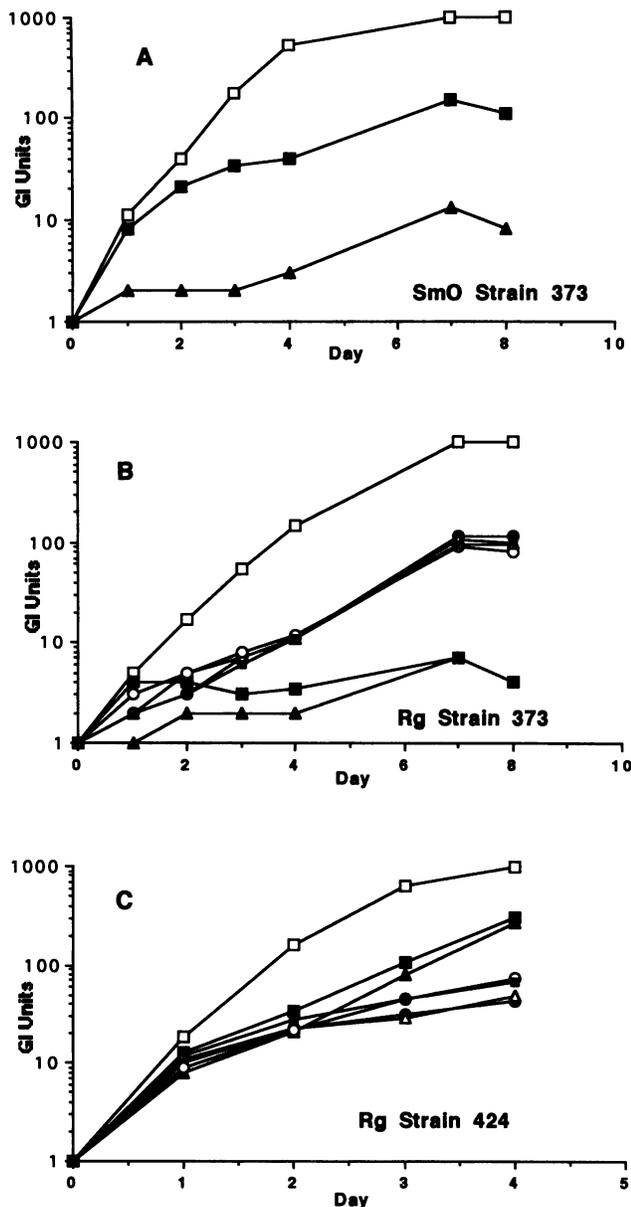


FIG. 4. Representative radiometric data showing the effects of various drug-inhibitor combinations on the growth of *M. avium*. (A) Amikacin (0.25  $\mu\text{g/ml}$ ) used in combination with either ethambutol (0.5  $\mu\text{g/ml}$ ) (■) or cerulenin (0.5  $\mu\text{g/ml}$ ) (▲) on *M. avium* SmO variant of strain 373 (control, □). (B) Sparfloxacin (4 mg/liter) used in combination with either ethambutol (0.5  $\mu\text{g/ml}$ ) (■), cerulenin (0.5  $\mu\text{g/ml}$ ) (▲), *N*-carbamyl-DL-phenylalanine (50  $\mu\text{g/ml}$ ) (●), *N*-carbamyl-L-isoleucine (100  $\mu\text{g/ml}$ ) (△), *trans*-cinnamic acid (10  $\mu\text{g/ml}$ ) (○), or *m*-fluoro-phenylalanine (50  $\mu\text{g/ml}$ ) (◻) on *M. avium* Rg variant of strain 373 (control, □). (C) Clarithromycin (0.5  $\mu\text{g/ml}$ ) used in combination with either ethambutol (0.5  $\mu\text{g/ml}$ ) (■), cerulenin (1.0  $\mu\text{g/ml}$ ) (▲), *N*-carbamyl-DL-phenylalanine (50  $\mu\text{g/ml}$ ) (●), *N*-carbamyl-L-isoleucine (100  $\mu\text{g/ml}$ ) (△), *trans*-cinnamic acid (100  $\mu\text{g/ml}$ ) (○), or *m*-fluoro-phenylalanine (50  $\mu\text{g/ml}$ ) (◻) on *M. avium* Rg variant of strain 424 (control, □). Growth is represented as GI values obtained with the BACTEC instrument and measured for 4 or 8 days following exposure to drug.

envelope is known to contain large quantities of complex lipids, it is very likely that cerulenin affects the architecture of the cell envelope, thus increasing its permeability for antimicrobial agents. Among these constituents would be the GPL and GPL-related components, which make up a large percentage of the cell envelope (4).

Examination of the HPLC patterns of [ $^{14}\text{C}$ ]Phe-labeled lipids revealed the presence of a component shared by all variants. Referred to as peak I, the component is apparently identical to a previously identified peak in other strains of *M. avium* (1, 5, 40, 41). This component is a nonglycosylated lipopeptide that contains phenylalanine, alanine, and isoleucine (5) and may play a role in GPL biosynthesis (40, 41). Also observed in the SmO variant of strain 373 was a cluster of phenylalanine-containing peaks with HPLC retention times a few minutes later than peak I. These apparently represent a major pool of components because of the amount of radiolabeled phenylalanine that was incorporated. Although not investigated in detail in the present study, it is very likely that at least one of these is similar to a glycosylated lipopeptide that we identified previously (1, 5, 40, 41). Because of the presence of phenylalanine, threonine, alanine, and alaninol, it most likely represents a precursor in GPL biosynthesis (5). Similar lipopeptides have been identified in *M. avium*, and they have been suggested to play a role in GPL biosynthesis (7).

Thus, as proposed previously (14), it appears that GPL biosynthesis proceeds by way of an initial fatty acyl-peptide core. It is likely that by inhibiting the biosynthesis of the lipopeptide cores it would be possible to inhibit initial events in GPL biosynthesis. Rg variants, which have greater quantities of these lipopeptides, would therefore be expected to be more susceptible to this type of treatment. This would in turn help to weaken the cell envelope and thus increase the activity of certain antimicrobial agents. Our results help to further confirm this rationale.

Using the internal radiolabeling techniques reported here, we examined four other species of mycobacteria for the presence of a peak similar to peak I. We were not able to identify such a peak in strains of *M. phlei*, *M. smegmatis*, or *M. goodii*; however, we were able to identify a phenylalanine-isoleucine-containing component in a strain of *M. paratuberculosis* by incorporation of [ $^{14}\text{C}$ ]Ile and [ $^{14}\text{C}$ ]Phe and analysis by HPLC (data not shown). A similar lipopeptide has been identified in *M. paratuberculosis*, but the ratio of phenylalanine:isoleucine:alanine was reported to be 2:2:1 (24), which is slightly different from that of peak I, which is 2:1:2, respectively (5). Peak I represents a major lipopeptide in Rg strains lacking serovar-specific GPL. This may be a partial explanation for their increased susceptibilities to inhibitory drugs with structural similarity to phenylalanine and isoleucine (5; this paper). Complete structural analysis of this lipopeptide is being conducted.

With regard to the three colony variants of strain 373, it appears that only the SmT variant contains serovar-specific GPL; this is usual. However, the fact that the SmO variant does not contain TLC-detectable serovar-specific GPL is surprising. To our knowledge, this has not been reported previously. The SmO variant of strain 373 does contain the serovar-nonspecific apolar GPL, as evidenced by HPLC and TLC, and may be a mutant which can be used in discerning the GPL biosynthetic pathway. Further studies are necessary to confirm that assumption. The absence of serovar-specific GPL in the Rg variant was expected because of previous studies (2, 6); however, Rg variants can still synthesize the lipopeptide core portion of the GPL (5, 7, 40).

It is interesting that of the three variants of strain 373, the

MICs of the antimicrobials were greater for the SmT and Rg variants than for the SmO variant, and likewise, the MICs of the antimicrobial agents for the Rg variant of strain 424 were greater overall than those for the SmO variant of strain 373. In addition, it is important to point out the greater susceptibilities of the Rg variants to combined treatments that include antimicrobial agents and analogs of certain amino acids. This may be important with regard to mixed infections in hosts. Although polyclonal infections (i.e., mixed serovars) have been reported previously, to our knowledge there are no reports of mixed colony types of the same strain being isolated from the same site. Therefore, there is no information regarding the evolution of morphotypes in patients. The existence of multiple morphotypes in the same patient is an important point that should be considered in the development of drug regimens for the treatment of *M. avium* infections. This may be one explanation for the range of drug susceptibility patterns and the increase in MICs in monotherapy trials (18) that have been reported in *M. avium*-infected patients.

From the results of the studies discussed here we can suggest two potential sites to which inhibitory drugs useful in the treatment of *M. avium* should be directed in the future. For all strains that we have examined (5; this paper), cerulenin has consistently shown low MICs and increased levels of activity when used in combination with antimicrobial agents at sublethal concentrations. Cerulenin is an inhibitor of fatty acid synthesis but is not useful as an antimicrobial agent because it is metabolized rapidly in the host (25, 26). Even so, the data in this paper suggest that the fatty acid synthetase system would be a plausible biosynthetic target for drugs developed in the future.

With regard to *M. avium* Rg variants, a potential target for drugs developed in the future appears to be peptide biosynthesis. In all the Rg strains that we tested (5; this paper), combined antimicrobial treatment with inhibitory drugs structurally analogous to phenylalanine has shown increased levels of activity, even sometimes when MICs are high (this paper). Although increased levels of activity with phenylalanine analogs have been demonstrated in the case of some Sm strains (5, 30, 32), the effects are not as dramatic as they are with Rg variants (5; this paper). Indeed, analogs of phenylalanine have been reported to inhibit GPL biosynthesis without inhibiting the growth of the organism (14). If the assumption that *M. avium* drug resistance is largely due to cell envelope impermeability is correct (13, 29), then these results suggest that the integrity of the cell envelope in the case of Rg variants depends more on components susceptible to phenylalanine-analogous drugs. The potential for lipopeptide biosynthesis being a good drug target is further supported by the results with drugs structurally analogous to isoleucine. Although the specific site for the action of these drugs is not yet known, it is plausible to suggest that the phenylalanine-isoleucine-containing lipopeptide may offer a reasonable explanation for that activity. All of these studies will have to be confirmed in a cell-free system (28) and are in progress.

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