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Differences in Antimicrobial Susceptibility of Pigmented and Unpigmented Colonial Variants of Mycobacterium avium

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Unpigmented colonial variants were isolated from pigmented Mycobacterium avium isolates recovered from patients with acquired immunodeficiency syndrome and the environment. The variants were interconvertible: the rate of transition from unpigmented to pigmented type was $4.0 \times 10^{-3}$ variants per cell per generation, and that from pigmented to unpigmented type was $1.5 \times 10^{-3}$ per cell per generation. The unpigmented variants were more tolerant to antibiotics, especially β-lactams, and Cd²⁺ and Cu²⁺ salts than were their pigmented parents. Both pigmented and unpigmented variants of the strains produced β-lactamase, although β-lactamase did not appear to be a determinant of β-lactam susceptibility. Pigmented variants grew more rapidly in a number of commonly used mycobacterial media, were more hydrophobic, and had higher carotenoid contents than their unpigmented segregants.

Materials and Methods

Mycobacterial strains. Four M. avium complex isolates (two clinical and two from soil) were selected for detailed study. They were chosen as representatives of 6 clinical and 20 environmental strains isolated and identified by our late colleague Howard Gruft of the New York State Department of Health, Albany. The two clinical M. avium isolates were obtained from patients with AIDS and designated strains 2812 and 2816, and the two soil isolates were designated strains W1158 and W1233. All were serotype 4 except strain W1233, which autoagglutinated. The strains were received as cultures of pigmented strains; upon culturing the pigmented strains were found to yield unpigmented segregants which yielded, in turn, pigmented colonies.

Growth of mycobacteria. Stock cultures were maintained on slants of Middlebrook (M) 7H9 broth base (BBL Microbiology Systems, Cockeysville, Md.) containing 1.5% (wt/vol) agar, 0.5% (vol/vol) glycerol, and 10% (vol/vol) oleic acid-albumin (OA) enrichment (20). For all experiments, except those employing the Sceptor System, single pigmented or unpigmented colonies were used to inoculate 2 ml of M7H9 containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OA enrichment (MGE broth), and cultures were incubated at 37°C until they reached log phase (4 to 7 days). The cells were harvested by centrifugation at 10,000 × g for 10 min at room temperature and suspended in buffered saline-gelatin (BSG; 0.85% [wt/vol] NaCl, 0.03% [wt/vol] K₂HPO₄, 0.06% [wt/vol] Na₂HPO₄, and 0.10% [wt/vol] gelatin) to an A₅₆₀ of 0.01 with a Colema Junior spectrophotometer (model 6A; Coleman Instruments, Maywood, Ill.). Preliminary experiments established that measurements at 580 nm removed any contribution of the yellow pigment to turbidity. The BSG suspensions were stored at 4°C.

Agar dilution MICs. A total of seven antimicrobial agents were incorporated into M7H10 agar medium containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OA enrichment (MGE agar) at concentrations ranging from 0.1 to 100 μg/ml. They were ciprofloxacin, ofloxacin, clofazimine, rifampin, rifabutin, and D-cycloserine. Cadmium (as CdCl₂) and copper (as CuCl₂) were incorporated into M7H10 containing only 0.5% (vol/vol) glycerol (MG agar) at concentra-
tions from 0.001 to 1.0 mM. *M. avium* complex strains were grown and suspended in BSG as described above and streaked on the surface of the media with a loop containing 0.001 ml of culture. One plate lacking antimicrobial agents was used as a control. Plates were incubated at 37°C for 14 days in plastic bags. *Escherichia coli* ATCC 25922 (28) was streaked onto the media immediately and after 14 days of incubation to test for deterioration of antibiotics. The MIC for the agar dilution method was defined as the lowest concentration of antimicrobial agent that yielded fewer than 10% of the number of colonies on the control plate.

**Broth microdilution MICs and MBCs.** Broth microdilution MICs and MBCs for the pigmented and unpigmented *M. avium* complex strains were determined by using the Sceptor Gram Positive panel and Sceptor Beta-Lactam Plus panel (Johnston Laboratories, Inc., Towson, Md.) as described by Yajko et al. (28). The MIC was defined as the lowest concentration of antimicrobial agent at which the organism showed no visible growth. The MBC (for acid-fast bacilli) was defined as the lowest concentration of drug that killed at least 99% of the original inoculum.

**β-Lactamase activity.** β-Lactamase activity was measured with the chromogenic cephalosporin pyridinium-2-azo-p-dimethylanilino chromophore (PADAC; Calbiochem-Behring, San Diego, Calif.) by a modification of the method described by Kobayashi et al. (10). Cells were grown in 60 ml of MGE broth medium with or without 0.01 μg of penicillin per ml to late log phase with shaking at 37°C. That penicillin concentration did not inhibit the growth of either strain. Cells were harvested by centrifugation (5,000 × g for 10 min at room temperature), the culture supernatant was saved (for β-lactamase measurement), and the cells were washed and suspended in 6 ml of 0.05 M potassium phosphate buffer (pH 7.0). β-Lactamase reaction mixtures contained 1 ml of 45 μM PADAC, 3 ml of 0.05 M potassium phosphate buffer (pH 7.0), and 1 ml of either the cell suspension or the culture supernatant in a screw-cap tube (16 by 25 mm) and were incubated at 37°C. A<sub>570</sub> was measured immediately and at 15-min intervals with a Coleman Junior spectrophotometer. β-Lactamase activity, reflected by PADAC hydrolysis and the loss of A<sub>570</sub>, was expressed as micromoles of PADAC hydrolyzed per hour per milligram (dry weight).

**Measurement of rate of colonial variation.** To measure the rate of colonial variation in strain 2812, a single colony of either strain 2812 P (pigmented) or strain 2812 U (unpigmented) was suspended in 2 ml of MGE broth in a screw-cap tube (16 by 125 mm) and incubated without shaking at 37°C. Immediately and at weekly intervals (up to 6 weeks), cultures of strain 2812 U were diluted in BSG and plated on MGE agar medium. After 4 weeks of incubation at 37°C, the numbers of pigmented and unpigmented colonies were counted. Cultures of strain 2812 P were grown and sampled in the same manner but were plated on MGE agar medium containing 2 μg of rifampin per ml. At this concentration of rifampin, only cells of the unpigmented variant grew (Table 1). Rates of colonial variation were calculated by the method of Witkin (26).

**Growth on solid media.** Growth of the pigmented and unpigmented variants was measured on the following solid media: MGE agar, MG agar, and a low pH minimal medium containing 1% (wt/vol) Tween 80 as the sole carbon source (TT agar [5]). BSG suspensions, prepared as described above, were streaked to the medium surface, and the medium was incubated at 37 or 43°C. Growth, scored as the presence or absence of single isolated colonies (≥1 mm in diameter), was recorded after 2 and 4 weeks of incubation.

For measurement of growth rates on Lowenstein-Jensen medium slants (BBL Microbiology Systems), slants were inoculated with 0.01 ml of a suspension of each strain grown and prepared as described above and were incubated at 37°C, and cells were harvested immediately and at weekly intervals by addition of 5 ml of BSG and suspension by vortexing. The suspensions were diluted in BSG and plated on MGE agar medium.

**Growth rates in liquid media.** Growth rates of pigmented and unpigmented *M. avium* complex strains were measured in the following broth media: MGE broth, M7H9 containing 0.5% (vol/vol) glycerol (MG broth), M7HSF (28), and Sauton medium. Sauton medium contained the following per liter: 4 g of l-asparagine, 2 g of citric acid, 60 ml of glycerol, 0.5 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of MgSO<sub>4</sub>, and 0.05 g of ferric ammonium citrate. The pH was adjusted to 7.2 with NH<sub>4</sub>OH before autoclaving. Growth rates, reflected by increases in turbidity, of 5-ml broth cultures containing 0.5 ml of inoculum in screw-cap tubes (16 by 125 mm) incubated vertically with shaking at 37 and 43°C were measured at 580 nm with a Coleman Junior spectrophotometer.

**Hydropophicity measurements.** Hydrophobicity was measured by adherence of cells to hexadecane as described by Ofek et al. (19). Strains 2812 P and 2812 U were grown on MGE agar medium for 7 days at 37°C, colonies of the opposite pigmentation were removed with a sterile glass tube, and cells were harvested by suspension in phosphate-buffered saline (19) with a sterile glass rod. The cell suspension was centrifuged at 10,000 × g for 10 min at room temperature, and cells were washed twice in phosphate-

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**TABLE 1.** Antibiotic and heavy metal susceptibilities of pigmented and unpigmented colonial variants of *M. avium* measured by agar dilution

<table>
<thead>
<tr>
<th>Antibiotic or heavy metal</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; for indicated <em>M. avium</em> strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2812</td>
</tr>
<tr>
<td></td>
<td>P U</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1 10</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>20 40</td>
</tr>
<tr>
<td>Clofazimine</td>
<td>0.2 0.5</td>
</tr>
<tr>
<td>Rifampin</td>
<td>&lt;1 &gt;40</td>
</tr>
<tr>
<td>Rifapentine</td>
<td>&lt;1 10</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>0.1 2</td>
</tr>
<tr>
<td>β-Cyclodexrine</td>
<td>20 20</td>
</tr>
<tr>
<td>Cadmium (Cd&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>0.1 1.0</td>
</tr>
<tr>
<td>Copper (Cu&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>0.1 &gt;1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC expressed as microgram of antibiotic per milliliter and millimolar concentration of heavy metal salt. P, Pigmented; U, unpigmented.
buffered saline and suspended at an A\textsubscript{580} of 0.5. A sample of the cell suspension (1.5 ml) was transferred to an acid-washed glass test tube (13 by 100 mm), and 0.05, 0.1, 0.15, or 0.20 ml of n-hexadecane (Sigma Chemical Co., St. Louis, Mo.) was added. The suspensions were vortexed at maximum speed for 2 min and left standing at room temperature for 20 min. The lower aqueous layer was removed, and its A\textsubscript{580} was measured (model 102 spectrophotometer; Hitachi Scientific Instruments, Tokyo, Japan). Results were expressed as the percentage of cells adherent to n-hexadecane (19). The hydrophobicity of the remaining strains was measured in the same way by using a single amount of hexadecane (0.10 ml).

To determine the effect of medium composition and stage of growth on hydrophobicity, cells of strains 2812 P and 2812 U were grown in MG broth, MGE broth, and M7HSF to log, late log, and early stationary phases and were harvested as described above. Hydrophobicity was measured as described above by using 0.10 ml of n-hexadecane. Each suspension was sampled to determine the frequency of colonial variants.

### Measurement of carotenoid content
The carotenoid content of strains 2812 P and 2812 U was measured by a modification of the method of Mathews (13) in cells grown for 2 weeks in 500 ml of MGE broth at 37°C. Cells were harvested by centrifugation at 10,000 \( \times \) g for 10 min and washed twice in water. To the washed cell pellet was added 5 ml of methanol, the suspension was vortexed, the cells were pelleted by centrifugation at 10,000 \( \times \) g for 10 min, and the yellow supernatant solution was saved in a foil-covered tube. The methanol extraction was repeated until the methanol was no longer yellow. The absorbance spectrum (300 to 600 nm) of the methanol extract was compared with those of \( \alpha \)- and \( \beta \)-carotene (Sigma) in methanol.

### Plasmid DNA isolation and characterization
The plasmid DNA content of each strain was determined by the method of Meissner and Falkinham (15).

### RESULTS

#### Antibiotic susceptibility patterns
The unpigmented variants of strains 2812, 2816, and W1158 were more resistant to ciprofloxacin, ofloxacin, cloramphenicol, rifampin, rifapentine, and rifabutin and to the heavy metals examined than were their pigmented parents (Tables 1 and 2). Although the antibiotic susceptibilities of the colonial variants of strain W1233 did not differ (Table 1 and 2), the unpigmented variant was more tolerant to both cadmium and copper (Table 1). For these experiments, the turbidity and CFUs per milliliter for the suspensions used for inoculation of each pair of colonial variants were the same.

The pigmented strains were also more susceptible to \( \beta \)-lactams than were their unpigmented segregants (Table 2). The increased tolerance for \( \beta \)-lactams of the unpigmented variants of strains 2812, 2816, and W1158 was not as great for newer, \( \beta \)-lactamase-resistant \( \beta \)-lactams, and the differences in susceptibility to \( \beta \)-lactams appeared to decrease when \( \beta \)-lactams were combined with clavulanic acid in the Sceptor Beta-Lactam Plus microdilution panel (Table 2). In addition, hydrolysis of nitrocefin was observed after 48 h of incubation at 37°C in the Sceptor Beta-Lactam Plus panels for all pigmented variants and unpigmented variants (data not shown). However, because of the nature of the test, those results were only qualitative.
Strain 2812 P was more susceptible to amikacin and erythromycin than were strain 2812 U and strain 2816 P to both erythromycin and clindamycin. Beyond those, there were no other significant differences between pigmented and unpigmented strains.

MBCs were also determined with the Sceptor microdilution panels as described by Yajko et al. (28). Only for strains 2812 P and 2816 P were 99% of the cells killed by concentrations of amikacin and ticarcillin-clavulanic acid (Timentin) achievable in serum (MBC/MIC ratio of 4 or less; data not shown). For the remaining strains and antibiotics, the MBC/MIC ratio was at least greater than 4, because the MBC was higher than the highest concentration of antibiotic in the Sceptor panel wells.

**TABLE 3. β-Lactamase activities of cells and culture supernatants of M. avium 2812 P and 2812 U**

<table>
<thead>
<tr>
<th>M. avium strain</th>
<th>Presence (+) or absence (−) of penicillin</th>
<th>Mean β-lactamase activity* ± SD of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells</td>
</tr>
<tr>
<td>2812 P</td>
<td>−</td>
<td>0.033 ± 0.007</td>
</tr>
<tr>
<td>2812 P</td>
<td>+</td>
<td>0.019 ± 0.004</td>
</tr>
<tr>
<td>2812 U</td>
<td>−</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>2812 U</td>
<td>+</td>
<td>0.016 ± 0.005</td>
</tr>
</tbody>
</table>

*Activity expressed as micromoles of PADAC hydrolyzed per hour per milligram (dry weight).

**Rate of transition.** The rate of appearance of pigmented colonies on MGE agar medium from cultures of strain 2812 U grown in MGE broth at 37°C was $4.0 \times 10^{-5}$ (±2.0 $\times 10^{-5}$) per 2812 U cell per generation. The rate of appearance of unpigmented colonies, measured as CFUs on MGE agar medium containing 2 μg of rifampin per ml, from cultures of strain 2812 P grown in MGE broth at 37°C was $3.2 \times 10^{-4}$ (±0.2 $\times 10^{-4}$) per cell per generation. The values represent the mean (plus or minus standard deviation) for four individual cultures and were corrected to account for the growth of preexisting pigmented and unpigmented CFUs in the initial cultures of 2812 U and 2812 P, respectively.

**Growth on solid media.** All colonial variants grew to form single isolated colonies on MGE agar in 2 weeks at either 37 or 43°C. The four unpigmented variants failed to form colonies on MG agar in 2 weeks at either 37 or 43°C, although their pigmented variants did form colonies. The unpigmented variants did form colonies by 4 weeks. None of the pigmented but all the unpigmented variants grew on the TT agar medium at 37°C in 4 weeks.

All pigmented and unpigmented variants grew on Lowenstein-Jensen medium slants; however, colonies of pigmented variants were visible while those of the unpigmented variants were not. To examine growth on Lowenstein-Jensen slants, the rates of growth of strains 2812 P and 2812 U were examined in detail. Not only did strain 2812 P grow faster (0.41 ± 0.13/day per generation) than strain 2812 U (0.52 ± 0.19/day per generation), the total number of cells recovered from slants after 2 weeks of incubation at 37°C was higher (7 $\times 10^{10}$ CFU per slant versus 1 $\times 10^{9}$ CFU per slant). However, those values were influenced by the fact that 30% of pigmented and 66% of unpigmented cells were recovered from slants after inoculation and immediate recovery.

**Growth rates in liquid media.** The growth of strains 2812 P and 2812 U was biphasic in MGE and Sauton media; initial growth (0 to 24 h) was rapid and then changed to a constant, lower rate (Fig. 1 and 2). Such biphasic growth was most likely caused by disruption of aggregates and fragmentation of multinucleate, filamentous cells (J. O. Falkingham, unpublished data). The generation times reported in Table 4 reflect the rates after that initial rapid phase. All four pigmented variants had higher growth rates than did their unpigmented variants at both 37 and 43°C in MGE broth, MG broth, and M7HSF (Table 4).

**Hydrophobicity measurements.** The hydrophobicity of cells of strain 2812 P was higher than that of strain 2812 U at all concentrations of hexadecane for cells grown on MGE agar plates (Fig. 3). For those experiments, the turbidity and cell number of the suspensions were equal (3 $\times 10^{7}$ CFU of 2812 P per ml and 4 $\times 10^{7}$ CFU of 2812 U per ml). On the basis of the results obtained with strain 2812, the hydrophobicity of the other strains grown on MGE agar was measured at a single concentration of hexadecane (0.1 ml). Cells of the other pigmented variants were also more hydrophobic than were their unpigmented derivatives (data not shown).

Hydrophobicity of cells of strains 2812 P and 2812 U grown to early log, mid-log, late log, and stationary phases in MGE, MG, and M7HSF broth media used for antimicrobial
susceptibility testing was also measured. Generally, cells of the pigmented variants were more hydrophobic than were those of the unpigmented variants, although both medium composition and stage of growth influenced the values (Table 5).

**Carotenoid content.** The absorption spectrum of methanol extracts of whole cells of the pigmented strain resembled that of β-carotene (data not shown). With \( A_{450} \), the carotenoid content of strain 2812 P was 10.2 μg of carotene per mg (dry weight), and that of strain 2812 U was 4.2 μg of carotene per mg (dry weight). Because the culture of strain 2812 U contained 30% pigmented CFUs, it is likely that the pigment content of the unpigmented variants is even lower than the value reported here. After extraction with methanol, extraction of cells with petroleum ether did not yield a yellow solution.

**TABLE 4. Growth rates of pigmented and unpigmented colonial variants of *M. avium***

<table>
<thead>
<tr>
<th><em>M. avium</em> strain</th>
<th>Growth rate* in indicated medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGE</td>
</tr>
<tr>
<td>2812 P</td>
<td>4</td>
</tr>
<tr>
<td>2812 U</td>
<td>7</td>
</tr>
<tr>
<td>2816 P</td>
<td>6</td>
</tr>
<tr>
<td>2816 U</td>
<td>14</td>
</tr>
<tr>
<td>W1158 P</td>
<td>6</td>
</tr>
<tr>
<td>W1158 U</td>
<td>14</td>
</tr>
<tr>
<td>W1233 P</td>
<td>7</td>
</tr>
<tr>
<td>W1233 U</td>
<td>10</td>
</tr>
</tbody>
</table>

* P, Pigmented; U, unpigmented.
* Growth rates expressed as number of days per generation at 37°C.
* Measured after first 24 h of incubation at 37°C.

**Plasmid DNA content.** Because previous reports demonstrated changes in plasmid DNA profiles coincident with changes in colonial morphology (4, 16), the plasmid DNA profiles of the pigmented and unpigmented variants were compared. No differences in plasmid DNA profiles between pigmented and unpigmented variants of the same strain were observed. Strain 2812 had two plasmids of 15.3 and 17.8 kilobases, strain 2816 had two plasmids of 15.3 and 19.4 kilobases, strain W1158 had two plasmids of 15.3 and 21.1 kilobases, and strain W1233 had a single plasmid of 15.3 kilobases.

**DISCUSSION**

Pigmented isolates of *M. avium* isolated from both patients with AIDS and the environment segregate unpigmented colony-forming variants, and the two forms are interconvertible. The rates of transition between the two forms was similar to the values reported for transitions...
between opaque and transparent colonial variants of *M. avium* (14, 27). The pigmented and unpigmented variants employed in this study were not unique, because independent pigmented variants of strain 2812 U (nine isolates tested) all shared the characteristic antibiotic susceptibility pattern of strain 2812 P. Growth in some media (i.e., Lowenstein-Jensen) resulted in the selection of one type over the other. Usually, the pigmented variant predominated in stationary-phase cultures, because it grew more rapidly (Table 4). Predominance in stationary-phase cultures could also be due to higher survival of one colonial variant in aging cultures, as was observed by Dunbar et al. (3). In addition, the bright yellow pigmentation would lead to more frequent detection and recovery of the pigmented colonial variant. This is especially the case with Lowenstein-Jensen medium, on which colonies of the unpigmented variant are barely visible. As a consequence of the appearance of colonies of the opposite type, cultures must be sampled to establish that the frequency of variants is low. In the experiments reported here (with the exception of those for measurement of carotenoid content), the percentage of cells of the opposite type was less than 1%

Not only were these *M. avium* strains relatively antibiotic resistant, as has been noted by others (7, 9, 29, 30), but the unpigmented colonial variants were significantly more antibiotic resistant than were the pigmented strains (Tables 1 and 2). Quite possibly, the unpigmented variants are analogs of the transparent colonial variants of *M. avium*, which are more antibiotic resistant than their opaque segregants (8, 11). In addition, the fact that the MBC/MIC ratios for the pigmented and unpigmented variants were greater than 4 for penicillins and cephalosporins suggests that these mycobacteria could be antibiotic tolerant (25). The combination of high-level resistance (i.e., a high MIC) and tolerance (i.e., a high MBC/MIC ratio) has been reported in other microorganisms (25).

The data provide the first proof of production of β-lactamase activity by strains of *M. avium* (Table 3). However, the activities of the strains do not provide an explanation for the differences in β-lactam and cephalosporin susceptibilities in pigmented and unpigmented colonial variants of *M. avium*. Whether production of β-lactamase is unique to this collection of clinical and environmental *M. avium* strains or is common among isolates awaits further testing.

One mechanism resulting in the relative antimicrobial resistance of unpigmented variants specifically, and *M. avium* strains in general, could be the existence of permeability barriers. The coincident acquisition of resistance to β-lactams, cephalosporins, aminoglycosides, macrolides, and heavy metals in the unpigmented derivatives of strains 2812 and 2816 (Table 2) suggests that the existence of a permeability barrier distinguishes strains 2812 U and 2816 U from their pigmented parents. Such permeability barriers have been proposed by others (17, 21).

The reduced hydrophobicity of strains 2812 U and 2816 U (Fig. 3; Table 5) could contribute to such a permeability barrier by reducing the ability of hydrophobic antibiotics to penetrate the outer layers. However, precise determination of whether differences in hydrophobicity are reflected in differences in drug susceptibility will require measurement of susceptibility to derivatives of drugs which share the same mode of action, yet whose hydrophobicities differ (12). In addition, because hydrophobicity is influenced by medium composition and growth stage (Table 5), in vitro antimicrobial susceptibilities might also be influenced by those two parameters.

The results also suggest that differences in growth rates of *M. avium* strains could contribute to differences in antibiotic susceptibility, as has been shown for other microorganisms (25). The growth rates of the pigmented, antibiotic-susceptible variants were higher than those of the unpigmented, antibiotic-resistant variants. Because inhibition of microorganisms by antibiotics is stronger in growing cultures (25), all or a fraction of the increased resistance of the unpigmented strains to antibiotics and heavy metals could be due to their lower growth rates.

The fact that the unpigmented variants are more resistant to antibiotics (Tables 1 and 2) than are their pigmented segregants suggests a reason for the failure of chemotherapy treatment of *M. avium* infections when in vitro susceptibility tests predicted drug susceptibility (7, 29, 30). It is possible that such tests have been performed with the more easily detected, antibiotic-susceptible pigmented variant. Antibiotic treatment would lead to the selection of drug-resistant, unpigmented variants. Because the frequency of transition is significantly higher than mutation and because the numbers of *M. avium* are quite high in cases of disseminated disease in patients with AIDS (≥10,000 CFU/ml of blood [29]), the frequency of drug-resistant, unpigmented variants may be quite high in patients.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


