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Decolorization of Malachite Green and Crystal Violet by Waterborne Pathogenic Mycobacteria

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***Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium marinum*, and *Mycobacterium chelonae* tolerate high concentrations of the dyes malachite green and crystal violet. Cells of strains of those species decolorized (reduced) both malachite green and crystal violet. Because decolorized malachite green lacked antimicrobial activity, the resistance of these mycobacteria could be due, in part, to their ability to decolorize the dyes. Small amounts of malachite green and its reduced, decolorized product were detected in the lipid fraction of *M. avium* strain A5 cells grown in the presence of malachite green, suggesting that a minor component of resistance could be due to sequestering the dyes in the extensive mycobacterial cell surface lipid. The membrane fraction of *M. avium* strain A5 had at least a fivefold-higher specific decolorization rate than did the crude extract, suggesting that the decolorization activity is membrane associated. The malachite green-decolorizing activity of the membrane fraction of *M. avium* strain A5 was abolished by either boiling or proteinase exposure, suggesting that the decolorizing activity was due to a protein. Decolorization activity of membrane fractions was stimulated by ferrous ion and inhibited by dinitrophenol and metyrapone.**

Malachite green and crystal violet are triphenylmethane dyes that are antimicrobial (1, 2, 6, 10, 14), toxic to mammalian cells (29), and mutagenic (3, 7, 12, 27). Malachite green has been used widely to prevent fungal infections in fish, typically at a concentration of 1 ppm (1, 2, 10, 18). Crystal violet is used to prevent fungal growth in poultry feed (6, 14), as a bacteriostatic agent in medical solutions (4, 24), and to treat skin infections by *Staphylococcus aureus* in humans and animals (23, 25). In addition the dyes are used as fabric and food dyes (2). Both malachite green and crystal violet can be reductively decolorized (2, 9, 15). Decolorized malachite green (i.e., leucomalachite green) was detected in liver and kidney tissue in rats after injection of malachite green (29). Intestinal microflora from a variety of animals have been shown capable of reducing malachite green (16) and gentian violet (21). Leucomalachite green is less toxic to both mammalian and bacterial cells than malachite green (12, 29), perhaps due to its insolubility in water (2, 9).

In contrast to most bacteria and fungi, mycobacteria are considered to be resistant to malachite green (2) and crystal violet (24). Many media for the cultivation of mycobacteria (e.g., Lowenstein-Jensen and Middlebrook 7H10) contain malachite green to reduce overgrowth by other, faster-growing microorganisms. Nosocomial infections due to *Mycobacterium chelonae* have been traced to the presence of that organism in a gentian violet skin-marking solution (24). A number of mycobacteria were described as being capable of "bleaching" malachite green (17), and a malachite green-reducing activity in acetone fractions of a number of rapidly growing mycobacteria has been described (26). Reduced malachite green was found in the lipid fraction of cells (26), consistent with its insolubility in water (2). Those results suggest that mycobacterial resistance to malachite green and crystal violet could be due to the reduction of the dyes and the sequestering of the dyes in the lipid fraction of cells.

Multiple isolates of *Mycobacterium avium* (12 isolates), *Mycobacterium intracellulare* (8 isolates), *Mycobacterium scrofulaceum* (5 isolates), and *Mycobacterium marinum* (3 isolates) and single isolates of *Mycobacterium fortuitum*, *Mycobacterium abscessus*, and *M. chelonae* were used to assess the levels at which malachite green and crystal violet were tolerated. Rates of decolorization were measured for strains *M. avium* A5 (28), *M. intracellulare* TMC 1406^T (20), *M. scrofulaceum* TMC 1323^T (20), *M. marinum* ATCC 11566, and *M. chelonae* M15 (11). Strains were grown in 100 ml of Middlebrook 7H9 broth medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (M7H9) in 500-ml nephelometer flasks to mid-log phase (7 days) at 37°C with aeration (60 rpm). Because of photooxidation of malachite green (1, 2, 19), flasks were covered with foil. Growth was measured as increases in turbidity (absorbance at 580 nm). Cells were collected by centrifugation (5,000 × *g* for 30 min at 25°C) and washed twice in chlorine demand-free phosphate buffer (CDFPB; pH 7) (8).

To measure malachite green and crystal violet susceptibility, cultures were streaked for isolated colonies by using a calibrated loop on M7H10 agar medium with different concentrations of malachite green and without malachite green. Following incubation at 37°C for 10 days, the lowest concentration abolishing colony formation was identified. Antimicrobial activity of malachite green, leucomalachite green, and mycobacterially decolorized malachite green was measured by a zone-of-inhibition assay (5). The results record the lowest concentrations of agents resulting in a 10-mm-diameter of zone of inhibition. Malachite green and crystal violet decolorization was measured in reaction mixtures that contained 5 μl of 0.1%

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(wt/vol) malachite green or 0.1% (wt/vol) crystal violet in CDFPB and 0.5 ml of cell suspension or cell fraction made up to a total volume of 1.0 ml with CDFPB. Fresh solutions of malachite green and crystal violet were prepared because spontaneous rates of decolorization were high. Experiments were performed under reduced light because of light-catalyzed reduction (1, 2, 19). Controls included the dyes alone or cells alone in CDFPB. Immediately after mixing the contents and at 5-min intervals, the absorbance at 620 nm (malachite green) or 580 nm (crystal violet) was measured. The decolorization rates were expressed as micrograms of dye decolorized per 10 min per milligram of dry weight of cells or milligram of protein of cell fractions at 25°C.

Measurement of malachite green and leucomalachite green in the lipid fraction of *M. avium* A5 cells was performed as described previously (26), and membrane and soluble fractions were prepared as described by George and Falkinham (13). Membrane fractions were boiled for 10 min or exposed to 1 mg of pronase (Sigma Chemical Co., St. Louis, Mo.) to measure the effects on malachite green decolorization rates. The effect of the presence of 1 mM NADH, 1 mM NADPH, 1 mM $FeSO_4 \cdot 7H_2O$, and 1 mM $FeCl_3$ was measured to determine whether those electron donors would accelerate or prolong the decolorization. The effects of the presence of the electron transport inhibitors 10 mM NaCN, NaN_3, dinitrophenol (DNP), and metyrapone (15) were measured to identify possible inhibitors of the decolorization reaction. The membrane fraction was incubated with the inhibitors for 10 min at 37°C before addition of malachite green.

All the mycobacterial strains tested were tolerant of malachite green and crystal violet above concentrations of 5 μg/ml. The median concentration preventing colony formation for all the mycobacterial strains was 60 μg/ml (range, 5 to 120 μg/ml) for malachite green and 15 μg/ml for crystal violet. There were no significant differences in the medians or ranges of dye susceptibilities for the different species or strains. Further, all the mycobacterial strains tested decolorized malachite green. Crystal violet decolorization was noted for many but not all strains, possibly because the inhibitory concentrations were below that where decolorization around the colonies could be detected.

Mycobacterially decolorized malachite green was produced by incubating a 30-μg/ml solution overnight in the dark at 37°C with 10^9 cells of *M. avium* strain A5 (1 mg [dry weight]) and filter sterilized. Both the mycobacterially decolorized malachite green and leucomalachite green lacked antimicrobial activity compared to malachite green (Table 1).

To determine if malachite green was associated with the lipid fraction of the mycobacterial cell envelope (and thereby sequestered), *M. avium* strain A5 cells were grown in the presence of 5 μg of malachite green/ml, 5 μg of crystal violet/ml, or neither (control). Although the amounts were small, both malachite green (i.e., 1 μg/mg [dry weight] of cells) and leucomalachite green (10 μg/mg [dry weight] of cells) could be detected in the lipid fraction. Crystal violet-grown cells also contained crystal violet (i.e., 2 μg/mg [dry weight] of cells) in the acetone extract.

Washed cells of *M. avium* strain A5, *M. intracellulare* strain TMC 1406^T, *M. scrofulaceum* strain TMC 1323^T, *M. marinum* strain ATCC 11566, and *M. chelonae* strain M15 all decolorized malachite green and crystal violet. There were no significant differences between the rates of decolorization of malachite green or crystal violet for cells of the five strains tested. The average decolorization rates ± standard deviations for the five strains were 0.020 ± 0.009 μg of malachite green decolorized in 10 min per mg dry weight at 25°C and 0.007 ± 0.003 μg of crystal violet in 10 min per mg of dry weight at 25°C. Two factors contributed to the variation in rate values. First, almost all mycobacterial cells aggregate and changes in aggregation occurred during measurement of decolorization and influenced absorption at 620 and 580 nm. Second, light intensity varied in spite of precautions to reduce light, resulting in different background rates. Decolorization rates at 25 to 30 min were approximately equal to those at 0 to 5 min, indicating that energy or cofactors for the decolorization (reduction) were not exhausted in 30 min. Decolorization of both dyes was evidently constitutive, because growth of *M. avium* strain A5 in subinhibitory concentrations of malachite green (0.25 μg/ml) did not increase the rates of decolorization or the concentration of dye preventing colony formation.

Average rates of decolorization of both dyes by crude extract, membrane, and soluble fractions of *M. avium* strain A5 are shown in Table 2. Both the crude extract and membrane fractions decolorized malachite green and crystal violet. The decolorization rate for the membrane fraction was significantly higher than that for the crude extract for both malachite green (5-fold) and crystal violet (16-fold). The rate of decolorization of malachite green by the soluble fraction was below the limit of measurement. The rate of crystal violet decolorization was lower than the rate of malachite green decolorization in both the crude extract and membrane fractions (Table 2), consistent with the lower rate of crystal violet decolorization by cells of *M. avium* strain A5 (data not shown). The decolorization of mal-

TABLE 1. Antimicrobial activities of malachite green, leucomalachite green, and mycobacterially decolorized malachite green

Target microorganism	Lowest concn (μg/ml) yielding a 10-mm zone of clearing		
	Malachite green	Leucomalachite green	Mycobacterially decolorized malachite green
Micrococcus luteus	4	>250	>30
Saccharomyces cerevisiae	1	125	>30
Candida albicans	8	>250	>30
Cryptococcus neoformans	0.5	125	>30
Aspergillus niger	16	>250	>30

TABLE 2. Rates of malachite green and crystal violet decolorization by cell fractions of *M. avium* strain A5

Cell fraction	Decolorization rate[a] ± SD	
	Malachite green	Crystal violet
Crude extract	0.64 ± 0.17	0.12 ± 0.02
Soluble	<0.08	ND[b]
Membrane	3.04 ± 0.43	1.90 ± 0.21

[a] Rates are expressed as micrograms of malachite green or crystal violet decolorized in 10 min per milligram of protein at 37°C.

[b] ND, not done.

TABLE 3. Effect of boiling and proteinase exposure and addition of electron donors and inhibitors of electron transport on the rate of malachite green decolorization by the membrane fraction of *M. avium* strain A5

Treatment	Malachite green decolorization rate (%)[a] ± SD
None	100
Boiling (10 min at 100°C)	<2 ± 0.5
Pronase (1 mg for 60 min at 37°C)	<2 ± 0.2
Fe^{3+} (1 mM)	110 ± 15
Fe^{2+} (1 mM)	157 ± 21
NADPH (1 mM)	90 ± 5
NADH (1 mM)	135 ± 12
NaCN (10 mM)	116 ± 7
NaN_3 (10 mM)	108 ± 5
DNP (10 mM)	<3 ± 0.5
Metyrapone (10 mM)	<3 ± 0.8

[a] Average of three separate measurements of two different membrane fractions.

achite green and crystal violet by membrane fractions was detected only for the first 15 min of incubation. After 15 min, the reaction halted, unlike decolorization by whole cells. That behavior suggests that some component (electron donor?) was being depleted from the membrane fraction. As was the case for whole cells, the rate of decolorization of either malachite green or crystal violet was not changed upon incubation under anaerobic conditions achieved by bubbling nitrogen gas through the reaction mixtures (data not shown).

Both boiling and pronase exposure abolished the ability of membrane fractions of *M. avium* strain A5 to decolorize malachite green (Table 3). Ferrous iron (1 mM) marginally increased the rate of decolorization by the membrane fraction (Table 3) and permitted the decolorization reaction to proceed beyond 15 min (data not shown). Only DNP (10 mM) and metyrapone (10 mM) inhibited decolorization by the membrane fraction (Table 3).

In confirmation of earlier anecdotal reports (2, 24), here we report the very high malachite green and crystal violet tolerance of a variety of mycobacterial species. The concentrations of malachite green and crystal violet tolerated by the mycobacteria are higher than those inhibitory to most other microorganisms (1, 6, 10, 18, 25). Thus, malachite green and crystal violet can serve as selective agents for the isolation of mycobacteria from samples (e.g., sputum or soil) that contain other, faster-growing microorganisms. This is a particularly valuable tool for recovering mycobacteria from patient and environmental samples in the presence of a competing microflora.

Neither mycobacterially decolorized malachite green nor leucomalachite green displayed antimicrobial activity (Table 1). Lanzing (18) had earlier shown that spontaneously decolorized malachite green (presumably a mixture of leucomalachite green and the carbinol form of malachite green [2, 9]) lacked antibacterial activity. Because decolorized gentian violet was shown to lack mutagenic activity (3), it is likely that the same holds true for malachite green. Thus, the decolorization of malachite green and crystal violet by mycobacteria would be expected to abolish the mutagenicity of the dyes.

The absence of antimicrobial activity of the mycobacterially decolorized malachite green suggests that at least one mechanism of mycobacterial resistance to the dye is decolorization. Earlier, it had been shown that decolorized gentian violet was less toxic to *Salmonella enterica* serovar Typhimurium cells (3). Although the rates of malachite green and crystal violet decolorization by the mycobacterial cell suspensions appear low, they are comparable to rates exhibited by intestinal bacteria (16, 21). In fact, 1 mg (dry weight) of cells (10^9 cells) was sufficient to decolorize 30 μg of malachite green in the absence of exogenous energy sources after overnight incubation at 37°C. Not only can mycobacteria grow in the presence of toxic concentrations of malachite green or crystal violet, but also it is likely that their ability to decolorize and inactivate the dyes permits the survival and growth of triphenylmethane dye-sensitive microorganisms in their environment, such as biofilms where *M. avium* and *M. intracellulare* are found (11).

Crude extracts and membrane fractions, but not the soluble fraction, of *M. avium* strain A5 (Table 2) decolorized malachite green and crystal violet. Based on the higher specific activity of the membrane fraction for both malachite green and crystal violet decolorization, it appears that the activity was enriched in that fraction of *M. avium* cells (Table 2). The data are consistent with evidence that a microsomal fraction of rat livers catalyzed the reduction of gentian violet (15). Although the malachite green-reducing activity was present in acetone extracts of rapidly growing mycobacteria, it was not localized to any specific cell fraction (26). Although the rates for decolorization of malachite green were higher than those for decolorization of crystal violet, we do not think that two different enzymatic activities were involved. First, the dyes are closely related. Second, crystal violet is more toxic than malachite green (3, 6) and the lower rate of crystal violet decolorization could reflect that dye's toxicity for the enzyme.

The sensitivity of decolorization by the membrane fraction to boiling and proteinase treatment (Table 3) suggests that the membrane fraction contains a protein that is responsible for both malachite green and crystal violet decolorization. Because the rate of malachite green decolorization by the membrane fractions fell to zero after 15 min of incubation, it was thought that the electron donor for the reaction was exhausted. Neither 1 mM NADH nor NADPH increased the rate or duration of the reaction (Table 3). That result differs from data showing that the activity of the soluble mycobacterial mercuric reductase was dependent on addition of either NADH or NADPH (22). Evidence that ferrous ion (1 mM) increased the rate of (Table 3) and prolonged decolorization suggests that the reaction was limited by the availability of reduced iron. Because malachite green decolorization by whole cells continued well beyond 30 to 60 min, cells could be reducing ferric iron. Decolorization was not inhibited by either azide or cyanide but was inhibited by DNP and metyrapone (Table 3). Inhibition by DNP suggests that decolorization requires hydrogen ion transfer across the membranes, and inhibition by metyrapone suggests that a cytochrome P-450 is involved in the reduction. Metyrapone inhibited reduction of gentian violet by rat hepatic microsomes (15). Because the decolorization reactions were performed in air, it is unlikely that the tri-(*para*-dimethylamino phenyl)methyl free radical was formed by reduction, because that reaction occurs only in the absence of oxygen (15). One objective of our continuing investigations is to identify the

electron acceptors for the decolorization and the membrane protein(s) and cofactors involved in decolorization.

A second factor contributing to the resistance of mycobacteria to malachite green and crystal violet is the sequestration of the dyes in the lipid fraction of, presumably, the cell wall. *M. avium* strain A5 cells grown in the presence of either 5 μg of malachite green or 5 μg of crystal violet per ml contained detectable levels of the dyes in the acetone extract. However, the amounts in the lipid extract were approximately 1,000 times smaller than the amount in the medium. Therefore, the sequestering of the dyes in the lipid fraction may not be a major contributor to the ability of *M. avium* strain A5 to grow in the presence of these dyes. Unfortunately, earlier reports did not provide the concentration of malachite green in the acetone extracts (26), so direct comparisons of the amounts could not be made.

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