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Effects of Biocides and Other Metal Removal Fluid Constituents on *Mycobacterium immunogenum*▽

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Cells of *Mycobacterium immunogenum* are significantly more resistant to biocides and antimicrobial compounds used for disinfection of metal removal fluids (MRFs) than cells of the gram-negative bacterium *Pseudomonas pseudoalcaligenes*. To ensure accurate measurement of *M. immunogenum* susceptibility, a biocide inactivation step must be included to prevent an overestimation of killing for short-term exposures. Cell suspensions in 5% MRF for 30 min rather than direct plating following biocide exposure increased the killing of *M. immunogenum* cells, suggesting a heretofore undiscovered interaction between MRF components and biocides. Biocide killing was reduced at high *M. immunogenum* cell densities (>10^6/ml). Among the chemicals tested, only Synergex Premier, Preventol CMK, and dicyclohexylamine killed greater than 90% of *M. immunogenum* cells in 6 h. *M. immunogenum* cells adhered to and grew rapidly on glass, copper, and galvanized surfaces, forming biofilms of 10^6 cells/cm^2 within 2 weeks and suggesting it is likely that the majority of *M. immunogenum* cells in MRF systems are on surfaces. *M. immunogenum* cells grown on surfaces in biofilms were three- to 100-fold more resistant to the biocides Synergex Premier and Preventol CMK than cells grown in suspension, compounding the problem of eradication of *M. immunogenum* cells from MRF systems.

It has been estimated that 1.2 million workers in the United States are exposed to aerosols generated by metal grinding and machining (20). Metal removal fluids (MRFs) are widely used in a variety of common industrial metal-grinding operations to carry off metal particles and lubricate and cool both the tool and the removal surface. MRFs typically consist of oil-in-water emulsions with additives such as emulsifiers, corrosion inhibitors, defoamers, and biocides (14). It has been proposed that exposure to MRF aerosols can lead to hypersensitivity pneumonitis (HP) (3, 16, 20, 26). A nontuberculous mycobacterial species, *Mycobacterium immunogenum*, has been recovered from a variety of MRFs and implicated as a possible cause of HP (20, 32). Supporting that hypothesis are data showing that mycobacteria were recovered more frequently from MRF samples collected from facilities where HP was found than those where it was absent (20, 26). The plausibility of HP following exposure to mycobacteria is supported by evidence that household exposure to *Mycobacterium avium* is associated with HP (18).

HP appeared in workers in spite of disinfection of the MRFs with morpholine, formaldehyde, or quaternary ammonium-based disinfectants (3, 26), and mycobacteria were recovered from the disinfected MRF (20, 32). Furthermore, it is well known that mycobacteria are resistant to short-term exposure to formaldehyde and quaternary ammonium disinfectants (4, 8, 30). *M. immunogenum* grew in some samples of spent, but not fresh, MRF (20). Growth might have been due to the ability of mycobacteria to grow on the organic compounds in MRF as has been shown for bacteria isolated from MRF (6). Mycobacteria can metabolize paraffins, pine oils (17), and naphthalene (13), as well as the disinfectant morpholine (9). Furthermore, disinfection may kill other competing microorganisms, allowing mycobacteria to predominate, as is the case for chlorine treatment of water (22). Mycobacteria readily form biofilms (2, 7, 23, 27), and mycobacterial cells in biofilms are more resistant to disinfection (27). Regrowth of surviving cells in biofilms is likely the reason why the addition of disinfectant and extensive cleaning of MRF delivery systems did not prevent the reappearance of mycobacteria (21, 31).

Because of the ease of use of biocides in reducing microbial numbers in MRF, herein we report the susceptibilities of strains of *M. immunogenum* and *Pseudomonas pseudoalcaligenes* (a gram-negative control) to a variety of biocides, expanding upon earlier studies (24, 28). In addition to examining the effect of growth stage and cell density, we report the biocide susceptibility of biofilm-grown *M. immunogenum*.

MATERIALS AND METHODS

Microbial strains. The following strains were used in the study: *Mycobacterium immunogenum* strains ATCC 700505^T and ATCC 700506 (32) and *Pseudomonas pseudoalcaligenes* strain ATCC 12815.

Biocides. The following MRF constituents and biocides were used: (i) the formaldehyde-releasing biocide Grotan [hexahydro-1,3,5-Tris(2-hydroxyethyl)-s-triazine; Troy Chemical Co., Ltd., Concord, Ontario, Canada]; (ii) the amines dicyclohexylamine (DCHA; Mallinckrodt Baker, Inc., Phillipsburg, NJ), AMP-95 (2-amino-2-methyl-1-propanol; Angus Chemical Company, Buffalo Grove, IL), Synergex (alkylamino alcohol; Arkema, Inc., Philadelphia, PA), Synergex T Plus (alkanolamine; Arkema, Inc.), and Synergex Premier (fatty amino alcohol; Arkema, Inc.); (ii) the isothiazolones Kordek LX5000 (2-methyl-4-isothiazol-3-one; Rohm and Haas, Philadelphia, PA), Kathon CC (5-chloro-2-methyl-4-isothiazol-3-one and 2-methyl-4-isothiazolin-3-one, Na_2Cu citrate; Coolant Control, Inc., Cincinnati, OH), and Kathon 886 MW (5-chloro-2-methyl-4-isothiazol-3-one, 2-methyl-4-isothiazolin-3-one, $CuNO_3 \cdot 3H_2O$; Coolant Control, Inc.); and (iv) the phenolics Preventol CMK (*p*-chlorometacresol; Lanxess Corp., Pittsburgh, PA) and Preventol O Extra (*o*-phenylphenol; Bayer Corp., Pittsburgh, PA). DCHA is a corrosion inhibitor used in certain semisynthetic MRFs but is reported to have antimicrobial activity (19). All results are reported as the concentration of the active agent in the products.

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Biocide-neutralizing broth. Difco D/E was used as the biocide-neutralizing broth (Difco, Detroit, MI) (11, 12).

MRF. The MRF used in the study was prepared by making a 5% (vol/vol) emulsion of a generic soluble oil (~75% naphthenic oil, 15 to 20% sodium sulfonate, ~5% coemulsifier) in sterile distilled water.

Growth of microorganisms in suspension. A single isolated colony was used to inoculate 10 ml of 1/10-strength Middlebrook 7H9 broth (*M. immunogenum*) or 1/10-strength tryptic soy broth (TSB) (*P. pseudoalcaligenes*) in a 125-ml flask. Cultures were incubated at 30°C with aeration (120 rpm) in a water bath. Growth was measured as increases in CFU on tryptic soy agar (*P. pseudoalcaligenes*) and Middlebrook 7H10 agar (*M. immunogenum*) after 2 and 7 days of incubation at 30°C (respectively). Cells of *M. immunogenum* were grown to exponential (2 weeks), early stationary (3 weeks), and stationary (4 weeks) phases as described previously (1). When cells reached the appropriate growth phase, reduced aggregate fractions (RAF) of the *M. immunogenum* cultures were prepared to remove large aggregates (29).

Growth of *M. immunogenum* in biofilms. *M. immunogenum* strain ATCC 700505^T cells were grown on 4-mm-diameter (0.50-cm^2 surface area) glass, copper, or galvanized steel beads in 1/10-strength M7H9 broth. Forty milliliters of 1/10-strength M7H9 broth containing 85 sterile beads in a 50-ml polystyrene, canted-neck tissue culture flask (Becton Dickinson Labware, Franklin Lakes, NJ) was inoculated with 4 ml of a culture of *M. immunogenum* strain ATCC 700505^T, and cultures were incubated at 30°C. Two hours after inoculation and after 1, 2, 3, and 4 weeks of incubation, five beads (in duplicate) were transferred from the flask to a petri dish containing 25 ml of sterile tap water using sterile tweezers, and the beads were washed by swirling for 30 s. To enumerate the number of cells on the beads, beads were transferred aseptically to a tube containing 1 ml D/E neutralizing broth, and the cells were separated from the beads by vortexing for 1 min at the highest speed. Undiluted and diluted suspensions (0.1 ml) were spread in triplicate on M7H10 agar and incubated at 30°C, and colonies were counted after 3 days.

Measurement of biocide susceptibility of suspended cells. Measurement of biocide killing of cells grown to different growth phases followed the standard practices of ASTM International (1) with the following additions: (i) RAF suspensions were employed for *M. immunogenum* cells (29) and (ii) biocides were neutralized with both D/E broth (11, 12) and 5% (vol/vol) MRF.

Measurement of biocide susceptibility of biofilm-grown *M. immunogenum*. After 2 and 4 weeks of incubation of beads in tissue culture flasks, 20 beads (in duplicate) were transferred from the flask to a petri dish containing 25 ml of sterile tap water using sterile tweezers, and the beads were washed by swirling for 30 s. The washed beads were then transferred to a beaker containing 20 ml of sterile tap water, and biocide was added to the desired final concentration (either 100 mg/liter Synergex Premier or 500 mg/liter Preventol CMK). Immediately and after 3, 6, and 24 h of incubation at 30°C, five beads were aseptically transferred to a tube containing 1 ml D/E neutralizing broth with a loop, and after a 30-min neutralization at 30°C, the cells were separated from the beads by vortexing for 1 min at the highest speed. Undiluted and diluted suspensions (0.1 ml) were spread in triplicate on M7H10 agar and incubated at 30°C, and colonies were counted after 3 days to calculate the surviving CFU/cm^2.

Effect of cell density on *M. immunogenum* biocide susceptibility. Cells of *M. immunogenum* strain ATCC 700505^T were grown to log phase in 100 ml of 1/10-strength M7H9 broth with aeration (60 rpm) at 30°C. RAF suspensions were prepared (29) and their turbidities adjusted to equal a no. 1 McFarland standard, and a 10-fold dilution series in 20 ml of sterile tap water (undiluted to 10^5) was prepared. Synergex Premier was added to each 20-ml suspension dilution to a final concentration of 100 mg/liter, and viable cell counts (CFU/ml) were measured at 0, 3, 6, and 24 h of incubation following neutralization in D/E neutralizing broth for 30 min at 30°C. Undiluted and diluted suspensions (0.1 ml) were spread in triplicate on M7H10 agar and incubated at 30°C, and colonies were counted after 3 days to calculate surviving CFU/ml.

RESULTS

Effect of biocide inactivation and MRF on biocide killing. To ensure that the cell killing was not influenced by carryover of biocide and killing on the agar medium, D/E neutralizing broth or 5% (vol/vol) MRF was used to inactivate the biocides before plating, as recommended by the standard practices of ASTM International (1). The principle is well established and is necessary because many biocides attach to cells and can remain attached during dilution operations and the transfer of cells to plates. In addition, dilutions may not be sufficient to reduce the concentration of biocide to below its killing (bactericidal) or inhibitory levels. The latter especially would be the case for the relatively biocide-susceptible cells of *P. pseudoalcaligenes*.

TABLE 1. Impact of biocide, D/E neutralization broth, and 5% (vol/vol) MRF on killing of *M. immunogenum* strain 700505^T cells

Biocide	Concn (mg active agent/liter)[a]	Postexposure treatment[a]	Fraction surviving after biocide exposure for indicated time[b]		
			3 h	6 h	24 h
Grotan	1,000	None	0.65	0.62	0.004
	1,000	D/E	0.76	0.63	0.012
	1,000	MRF	0.70	0.41	0.0017
AMP-95	100	None	1.05	1.07	0.31
	100	D/E	0.97	0.97	0.32
	100	MRF	1.00	0.74	0.10
Synergex Plus	100	None	0.82	0.71	0.27
	100	D/E	0.89	0.74	0.26
	100	MRF	0.65	0.53	0.14
Synergex Premier	100	None	0.13	0.047	<0.00072
	100	D/E	0.10	0.034	<0.00065
	100	MRF	0.017	0.0011	<0.00047
Preventol CMK	500	None	0.41	0.057	<0.0015
	500	D/E	0.65	0.12	0.005
	500	MRF	0.37	0.038	<0.0009

[a] Cells were suspended in D/E broth or 5% (vol/vol) MRF for 30 min following biocide exposure.
[b] Calculated as biocide-exposed CFU divided by control CFU for the same duration of exposure from an average of two independent experiments.

As illustrated by the data in Table 1, survival of *M. immunogenum* cells despite exposure to the biocides Preventol CMK (phenolic) and Grotan (formaldehyde releasing) was increased if biocide-exposed cells were suspended in the D/E neutralizing broth for 30 min before plating. Depending upon the duration of biocide exposure, the number of surviving cells was increased by as much as three- to fourfold (Table 1). D/E neutralization did not increase the survival of cells exposed to the amines AMP-95, Synergex T Plus, or Synergex Premier (Table 1). To ensure that carryover effects did not affect survival values, D/E neutralization was employed for all the following measurements.

Exposure of biocide-exposed cells of *M. immunogenum* to MRF. Because some of the components in the D/E neutralizing broth are also present in MRFs (e.g., fatty acids), the possibility that MRF could protect cells from biocide-induced killing was tested. Following exposure to biocide, samples were mixed with 5% (vol/vol) MRF and incubated for 30 min before plating. Rather than observing protection, we observed that survival was unchanged or moderately reduced except for Synergex Premier where survival was reduced (Table 1). It is likely that inhibition of *M. immunogenum* colony formation, especially after a 24-h exposure, could be related to the fact that *M. immunogenum* could not grow in the fresh 5% (vol/vol) MRF (J. O. Falkinham III, unpublished data).

Biocide susceptibilities of *M. immunogenum* and *P. pseudoalcaligenes*. The rate of *M. immunogenum* survival was higher than that of *P. pseudoalcaligenes* at the same biocide concen-

TABLE 2. Biocide killing of *P. pseudoalcaligenes* and *M. immunogenum* strains

Biocide	Concn (mg active agent/liter)	Fraction surviving after biocide exposure for indicated time[a,b]					
		P. pseudoalcaligenes 12815			*M. immunogenum* 700500^T		
		3 h	6 h	24 h	3 h	6 h	24 h
Grotan	1,000	0.0012	<0.00005	<0.00005	0.76	0.63	0.012
AMP-95	100	0.51	0.36	0.019	0.97	0.97	0.32
Synergex	100	0.037	0.014	0.033	0.91	0.88	0.66
Synergex Plus	100	0.020	0.018	0.78	0.89	0.74	0.26
Synergex Premium	100	0.0014	<0.0014	<0.0014	0.10	0.0034	0.00065
DCHA	250	ND	ND	ND	0.047	0.00076	<0.00069
Kathon CC	10	0.014	0.0014	0.00042	0.77	0.72	1.16
Kathon 886 MW	10	0.20	0.012	0.003	0.69	0.54	0.039
Kordek	125	0.33	0.06	0.002	0.87	0.68	0.024
Preventol O Extra	1,000	1.41	5.23	>15	>2	>2	>2
Preventol CMK	500	<0.001	<0.001	<0.001	0.65	0.12	<0.0014

[a] Cells were suspended in D/E broth for 30 min following biocide exposure.
[b] Calculated as biocide-exposed CFU divided by control CFU for the same exposure duration from an average of two independent experiments. ND, not done.

trations (Table 2). Most biocides, with the exception of Synergex Premier, DCHA, and Preventol CMK, were incapable of killing *M. immunogenum* cells in the short term (3 and 6 h) and long term (24 h) as measured by surviving colony counts (Table 2). Grotan was effective against *M. immunogenum* but only for long-term exposure (Table 2). Based on the survival values, 3, 5, and 7 h of exposure to Synergex Premier (100 mg/liter) or DCHA (250 mg/liter) would be required to kill 90%, 99%, and 99.5% of *M. immunogenum* cells. Lower dosages would be sufficient to kill *P. pseudoalcaligenes*. Preliminary experiments established that there was no difference in susceptibilities of the two *M. immunogenum* strains and that logarithmic and stationary phase cells of *M. immunogenum* did not differ in the pattern of biocide susceptibility. To rule out the possibility that the increased survival of *M. immunogenum* strains was due to their growth in 1/10-strength M7H9 broth, cells were also grown in 1/10-strength TSB. Those *M. immunogenum* cells grown in 1/10-strength TSB displayed the same biocide resistance as those grown in M7H9 broth (data not shown). The data in Table 2 reflect the susceptibility of the type strain of *M. immunogenum* (ATCC 700505^T) grown to exponential phase. Survival values were measured at single concentrations of biocides, namely, those recommended by the manufacturer. Biocides were not tested at higher concentrations because they either exceeded the solubility of the biocide or the recommended safety level (i.e., material safety data sheets).

Exposure of cells of both *P. pseudoalcaligenes* and *M. immunogenum* to Preventol O Extra led to increases in colony counts in both the short and long term (Table 2). The increase for *M. immunogenum* was higher than the 5 to 10% increase due to the separation of aggregated cells promoted by detergent action on the hydrophobic mycobacterial cells. Possibly, the *M. immunogenum* strain could have metabolized the biocide for growth. In the 1/10-strength medium, the time required for one doubling was 2.4 h. Thus, the 1.4- and fivefold increase in cell number is consistent with that growth rate. The increase in survival of *P. pseudoalcaligenes* after a 24-h exposure to Synergex Plus was reproducible (Table 2), but its basis is unknown.

Effect of cell density on biocide killing. Susceptibility of *M. avium* to chlorine is influenced by cell density; namely, reduced killing is observed at high cell densities due to the disappearance of chlorine (10). Because high cell densities could also inactivate and reduce the concentrations of biocides, the effect of high *M. immunogenum* densities on Synergex Premier killing was measured. The results demonstrate increased survival of *M. immunogenum* cells exposed to Synergex Premier at cell densities of $>10^6$ CFU/ml (Table 3). For the experiments reported here (Tables 1 and 2), the densities of cells exposed to biocides were between 10^3 and 10^5 CFU/ml, well below that critical cell density.

Growth of *M. immunogenum* in biofilms and biocide susceptibilities of biofilm-grown cells. Cells of *M. immunogenum* readily attached and increased in number in biofilms on the surfaces of glass, copper, and galvanized steel (Table 4). As beads were incubated in flasks containing cells growing in suspension, the increases in CFU/cm^2 reflect both growth of *M. immunogenum* cells in biofilms and continued attachment of cells from suspension. A higher survival rate was noted for biofilm-grown cells of *M. immunogenum* exposed to either Synergex Premier (Table 5) or Preventol CMK (Table 6) than cells grown in suspension. Interestingly, there was no increased survival of biofilm-grown *M. immunogenum* cells exposed to Preventol CMK after a 24-h exposure (Table 6) compared to that of cells grown and exposed in suspension (Table 2). Perhaps

TABLE 3. Increased resistance of *M. immunogenum* strain ATCC 700505^T to Synergex Premier (100 mg/liter) at high cell density

Expt	Cell density (cells/ml)	Fraction surviving after biocide exposure for indicated time[a,b]		
		3 h	6 h	24 h
1	1.5×10^6	0.27	0.037	<0.001
	1.3×10^5	0.17	0.022	<0.001
2	1.0×10^6	0.40	0.20	0.09
	9.0×10^4	0.14	0.02	<0.00003
3	4.5×10^6	0.38	0.23	0.013
	4.5×10^5	0.24	0.13	0.000007
	5.1×10^4	0.24	0.12	0.00008

[a] Cells were suspended in D/E broth for 30 min following biocide exposure.
[b] Calculated as biocide-exposed CFU divided by control CFU for the same exposure duration from an average of two independent experiments.

TABLE 4. Growth of *M. immunogenum* strain ATCC 700505^T cells on glass, copper, and galvanized beads

Incubation period or wk	CFU/cm^2 (fold increase) of cells grown on indicated surface[a]			
	Glass		Copper	Galvanized beads
	Expt 1	Expt 2		
Attachment	1.1×10^5	4.2×10^4	3.0×10^4	6.0×10^3
1	5.6×10^5	1.0×10^5	9.2×10^5	1.3×10^6
2	6.8×10^5	4.2×10^5	1.4×10^6	2.6×10^6
3	3.4×10^5	8.4×10^5	1.9×10^6	5.6×10^6
4	3.0×10^5 (2.5)	1.0×10^6 (24)	3.6×10^6 (120)	5.0×10^6 (833)

[a] Average of two independent experiments.

this represents a biocide diffusion limitation into the biofilm rather than an inherently greater biocide resistance due to growth in a biofilm.

DISCUSSION

Measurements of the susceptibility of *M. immunogenum* to biocides clearly demonstrate the relative resistance of this species to a wide range of biocides and metal removal constituents compared to *P. pseudoalcaligenes* as documented earlier by Selvaraju and colleagues (24, 25) and others (28). That result is not surprising based on the resistance of mycobacteria to formaldehyde and quaternary ammonium compounds (4, 8, 30) and chlorine, chloramine, chlorine dioxide, and ozone (29). Although here it is shown that *M. immunogenum* is susceptible to the formaldehyde-releasing product Grotan upon long-term exposure (24 h) (Table 2), this does not necessarily conflict with earlier reports demonstrating the formaldehyde resistance of mycobacteria as those results were based on short-term exposure at high density (30). Following completion of this study, it was discovered that there exist different *M. immunogenum* genotypes that differ over a twofold range in biocide susceptibilities (25) and could not be included in this study.

It does not appear that the biocide susceptibility of *P. pseudoalcaligenes* can be used as a surrogate to guide the choice of biocide for *M. immunogenum*. A comparison of the survival values of *M. immunogenum* and *P. pseudoalcaligenes* for nine different biocides (excluding DCHA) at the same dosage by linear regression showed that the correlation coefficient ($r^2 = 0.18$) was too low to be significant. Even reducing the biocides to the four amines (AMP-95, Synergex, Synergex T Plus, and Synergex Premier) did not improve the correlation coefficient ($r^2 = 0.21$) to the point where the *P. pseudoalcaligenes* data could be used to choose the biocide, concentration, and exposure duration for *M. immunogenum*.

TABLE 5. Susceptibility of *M. immunogenum* strain ATCC 700505^T cells to Synergex Premier (100 mg/liter)

Duration of exposure (h)	Fraction surviving after biocide exposure under indicated growth conditions[a,b]				
	2-wk suspension	2-wk biofilm		4-wk biofilm	
		Expt 1	Expt 2	Expt 1	Expt 2
0	1.00	1.00	1.00	1.00	1.00
3	0.10	0.38	0.44	0.85	0.29
6	0.002	0.36	0.23	0.54	0.25
24	<0.0006	0.002	0.002	0.012	0.009

[a] Cells were suspended in D/E broth for 30 min following biocide exposure.
[b] Calculated as biocide-exposed CFU divided by control CFU for the same exposure duration from an average of two independent experiments.

TABLE 6. Preventol CMK (500 mg/liter) susceptibility of *M. immunogenum* strain ATCC 700505^T cells grown for 4 weeks

Duration of exposure (h)	Fraction surviving after biocide exposure under indicated growth conditions[a,b]			
	Suspension	Glass biofilm	Copper biofilm	Galvanized bead biofilm
0	1.00	1.00	1.00	1.00
3	0.64	0.94	0.71	0.90
6	0.11	0.61	0.39	0.36
24	<0.0014	0.008	0.005	0.004

[a] Cells were suspended in D/E broth for 30 min following biocide exposure.
[b] Calculated as biocide-exposed CFU divided by control CFU for the same exposure duration from an average of two independent experiments.

Biocide neutralization with D/E broth increased the survival of the representative formaldehyde-releasing (i.e., Grotan) and phenolic-based (i.e., Preventol CMK) biocides (Table 1). Earlier, it was shown that albumin increased the survival of microorganisms exposed to isothiazol-based biocides (28). Accordingly, it is recommended that measurements of biocide killing include neutralization as a matter of course, following the standard practices of ASTM International (1). A measurement of killing performed in the absence of neutralization would overestimate the true killing in the laboratory and lead to the use of inadequate biocide dosages in the field. Thus, when evaluating biocides, it is important to know the methods and conditions under which the biocide killing was measured.

Microbial and mycobacterial cell densities in MRFs for use in automobile plants can be as high as 10^8 CFU/ml (20, 26). Unfortunately, at cell densities of $>10^6$ CFU/ml, biocide killing was reduced (Table 3). The reduced killing of *M. immunogenum* cells in biofilms may be due to the high cell concentrations (Table 4). Perhaps, as is the case for the chlorine killing of mycobacteria (10), high concentrations of cells lead to the disappearance or sequestering of the antimicrobial agent. Data are available on the susceptibilities of *Mycobacterium* spp. to high-density inocula (10^8 CFU/ml) (28). Furthermore, suggested biocide inhibitory concentrations used to guide addition of biocides to MRF may not be predictive of efficacy at densities above 10^6 CFU/ml. Methods are available for the rapid estimation of mycobacterial numbers in MRFs before they reach levels that prevent or reduce biocide efficacy (15, 28).

Two biocides, Synergex Premier and DCHA, exhibited 99.9% killing of *M. immunogenum* cells in short-term exposures (Table 2). Exposure of *M. immunogenum* cells for 24 h to DCHA, Synergex Premier, or Preventol CMK also resulted in ≥99.9% killing. DCHA is used as a corrosion inhibitor and data here also demonstrate its antimycobacterial activity. Both Synergex Premier and Preventol CMK killed >99% of *M. immunogenum* cells in biofilms (Tables 5 and 6), suggesting that they are capable of penetrating the extracellular matrix and cell layers of biofilms. Preventol CMK appears equally capable of killing cells in either suspension or biofilms (Table 6). Inasmuch as killing by either Synergex Premier or Preventol

CMK was increased upon exposure to fresh 5% (vol/vol) MRF (Table 1), their inclusion in programs for the reduction of mycobacterial numbers in MRF appears logical. Unfortunately, a detailed listing of the composition of Synergex Premier or Preventol CMK was not available in the material safety data sheets, preventing guidance for formulation of an antimycobacterial biocide.

Caution should be taken in extending the data presented here to implementation of programs to manage mycobacterial numbers in MRFs. Because of the failure of *M. immunogenum* to grow in fresh MRF (20), it was not possible to duplicate the physiological state of cells in MRF. The great variety in composition of MRFs (14) further compounds that problem. Although *M. immunogenum* cells can grow in spent or used MRF (20), each may have unique chemical and microbial compositions, thus limiting their utility as test substrates.

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