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ORIGINAL PAPER

Differential biocide susceptibility of the multiple genotypes of *Mycobacterium immunogenum*

Suresh B. Selvaraju · Izhar U. H. Khan · Jagjit S. Yadav

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Abstract The non-tuberculous mycobacterium *Mycobacterium immunogenum* colonizes industrial metalworking fluids (MWFs) presumably due to its relative resistance to the currently practiced biocides and has been implicated in occupational respiratory hazards, particularly hypersensitivity pneumonitis. With an aim to understand its inherent biocide susceptibility profile and survival potential in MWF, five different genotypes of this organism, including a reference genotype (700506) and four novel test genotypes (MJY-3, MJY-4, MJY-10 and MJY-12) isolated in our recent study from diverse MWF operations were evaluated. For this, two commercial biocide formulations, Grotan (Hexahydro-1,3,5-tris(2-hydroxyethyl)-*s*-triazine) and Kathon (5-chloro-2-methyl-4-isothiazolin-3-one) currently practiced for the control of microorganisms, including mycobacteria, in MWF operations were tested. Effect of the fluid matrix on the biocide susceptibility was investigated for the synthetic (S) and semi-synthetic (SS) MWF matrices. In general, the minimum inhibitory concentration values were higher for the HCHO-releasing biocide Grotan than the isothiazolone biocide Kathon. All genotypes (except the reference genotype) showed lower susceptibility in SS as compared to S fluid matrix for Grotan. However, in case of Kathon, a greater susceptibility was observed in SS fluid for majority of the test genotypes (MJY-3, 4 and 10). The test genotypes were more resistant than the reference genotype to either biocide in both fluid types. Furthermore, the individual genotypes showed differential biocidal susceptibility, with MJY-10 being the most resistant. These observations emphasize the importance of using the resistant genotypes of *M. immunogenum* as the test strains for formulation or development and evaluation of existing and novel biocides, for industrial applications.

Keywords Metalworking fluids · Biocides · *Mycobacterium immunogenum* · Non-tuberculous mycobacteria · Hypersensitivity pneumonitis · Antimicrobial resistance

S. B. Selvaraju · I. U. H. Khan · J. S. Yadav (✉)
Department of Environmental Health,
University of Cincinnati College of Medicine,
Cincinnati, OH 45267-0056, USA
e-mail: Jagjit.Yadav@uc.edu

Present Address:
S. B. Selvaraju
Children's Mercy Hospitals and Clinics,
2401 Gillham Road, 3719.00 Research,
Kansas City, MO 64108, USA

Present Address:
I. U. H. Khan
Environment Canada, National Water Research Institute (NWRI),
867 Lakeshore Rd, Burlington, ON L7R 4A6, Canada

Introduction

Environmental mycobacteria, particularly nontuberculous mycobacteria (NTM) have been implicated in outbreaks of hypersensitivity pneumonitis (HP) and other respiratory disorders in occupational, recreational, and hospital settings [5, 17]. In particular, two species of the *Mycobacterium chelonae* complex (MCC), namely *M. immunogenum* and *M. chelonae* have been reported from metalworking fluids (MWFs) that are associated with occupational exposures in industrial machine workers [6, 15, 22]. Lately, *M. immunogenum* has been implicated in the outbreaks of HP among machine workers exposed to the contaminated water-based MWFs [1, 9, 18, 21].

As a routine practice, different chemical biocides are added to control microbial growth in these water-based

machining fluids in industrial operations [3, 12]. Often, different biocides at various amounts, particularly formaldehyde-releasing biocides and isothiazolone biocides are applied to control various groups of microbial contaminants including mycobacteria [16]. These and other commercial biocides meant for the control of mycobacterial growth in MWF are developed and evaluated against standard laboratory test species/strains for mycobacteria such as laboratory strains of *M. smegmatis*. The recommended effective dose may therefore be different from the actual dose needed to control mycobacteria in actual applications. Consequently, these biocides are applied indiscriminately without prior testing of their efficacy towards specific MWF-associated species/strains of mycobacteria, thereby causing unwarranted toxicity-related potential health hazards in the exposed workers. This warrants evaluation of the efficacy of the newly developed biocides or existing potentially effective biocides on actual species/strains of mycobacteria prevalent in these fluids. This will enable their judicious use in an industrial setup and help understand an optimal test inoculum for development and efficacy testing of biocides meant for mycobacteria control. The aim of this study was to investigate the relative biocidal susceptibility of five different genotypes of *M. immunogenum* species (originally isolated from contaminated industrial MWF samples) against two commonly applied biocide formulations, Grotan (HCHO-based) and Kathon (Isothiazolone based) in terms of the minimum inhibitory concentration (MIC) as tested in two MWF fluid matrices, the synthetic (S) and semi-synthetic (SS). To our knowledge, this is the first report on investigation of the effect of any biocide against the actual isolates/genotypes of MWF-associated mycobacterium and is expected to help improve the existing practices of biocide formulation and biocide regime for controlling mycobacteria in industrial metalworking fluids.

Materials and methods

Microbial strains and culture conditions

Mycobacterium immunogenum reference genotype (ATCC 700506) and four novel genotypes (MJY-3, MJY-4, MJY-10 and MJY-12) recently isolated in our laboratory [6] were used as the test genotypes. All experiments were performed using M7H9 broth and M7H10 agar (Difco Laboratories, Sparks, MD), both supplemented with 10% Oleic acid–Albumin–Dextrose–Catalase enrichment (OADC, BD Biosciences, Sparks, MD) and 0.5% glycerol. The different genotypes of *M. immunogenum* were grown to exponential phase in 40 ml of broth with continuous shaking (150 rpm) at 37°C for 120 h to a cell density equivalent to 120 Klett reading ($\sim 10^8$ CFU/ml) measured using Klett Photoelectric Colorimeter (Klett, New York, NY).

Biocides and metalworking fluids

Two commercial biocides were evaluated, including one formaldehyde (HCHO)-releasing biocide, Grotan with hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine, 78.9% as active ingredient (Troy Chemical Corp., Newark, NJ) and an isothiazolone group biocide, Kathon 886 MW, with 5-chloro-2-methyl-4-isothiazolin-3-one, 14.1% as active ingredient (Rohm & Haas Company, Philadelphia, PA). The commercial synthetic and semi-synthetic metalworking fluids at the commonly practiced working concentrations of 5% (v/v) and 2% (v/v), respectively were filter sterilized and used as the test matrices.

Biocide efficacy test

Efficacy of the two test biocides was studied in fluid suspension tests in terms of logarithmic reductions in viable count (colony forming units, CFU/ml) of the test organisms, using different concentrations of the test biocide and a 60 min contact time. The log reductions were estimated by converting initial and post-treatment bacterial counts (CFU/ml) to $\log_{10}$ values and subtracting the mean of the final $\log_{10}$ values from the initial $\log_{10}$ value. The initial log value was obtained from the mean of the values for the control containing no biocide.

The test organisms were diluted to attain 10^8 CFU/ml in MWF matrices. One ml aliquots of the cell suspension of individual test organisms were placed into 1.5 ml micro centrifuge tubes and treated with individual biocides. A single biocide was added at varying final concentrations (100 through 100,000 ppm) to the test suspension, followed by incubation at room temperature for 60 min. The test concentration range for the biocides varied between 100 and 100,000 ppm based on our prior experience [16] with these biocides. Survival of the test organisms was determined by taking 100 µl aliquot from each treatment and spread plating on M7H10 agar followed by incubation at 37°C for 120 h. The MIC value corresponded to the minimum biocide concentration and contact time combination that resulted in 100% inactivation of the test organism in a given test MWF matrix. The inactivation was measured in terms of complete loss of cultivability of the test organism as determined by spread plating.

Results

Efficacy of the HCHO-releasing biocide (Grotan) toward different genotypes of *M. immunogenum*

The results showed that the four test genotypes of *M. immunogenum*, originally isolated from in-use MWF in our

recent studies, are less susceptible to Grotan than the reference genotype particularly in semi-synthetic (SS) fluid as compared to the synthetic (S) fluid matrix (Fig. 1). In general, MJY-4 and MJY-10 were observed to be the least susceptible to Grotan among the genotypes and the following overall trend was observed for genotype susceptibility to Grotan: reference genotype > MJY-12 = MJY-3 > MJY-4 > MJY-10. The individual genotypes showed variable rates of log reduction as the test biocide concentration increased.

In semi-synthetic fluid, the reference genotype showed a relatively rapid rate of inactivation than the other test genotypes, resulting in complete inactivation at 20,000 ppm concentration of this biocide (Fig. 1a). MJY-3 and MJY-12 genotypes showed a gradual increase in log reduction for every 10,000 ppm increase in the biocide level and reaching complete inactivation at 50,000 ppm concentration whereas the more resistant genotypes MJY-4 and MJY-10 showed none or a slow rate of inactivation in the 10,000–50,000 ppm concentration range (Fig. 1a).

In synthetic fluid matrix, a gradual initial log reduction (1–2 logs) in cell number was observed for every 10,000 ppm increase in biocide concentration, followed by more rapid reduction in case of the reference, MJY-3 and MJY-12 genotypes (Fig. 1b). In contrast, MJY-4 and MJY-10 genotypes showed the lowest rate of reduction and survived even at the high-test concentrations (up to 40,000 ppm) of the biocide (Fig. 1b).

Efficacy of the Isoathiazolone biocide (Kathon 886 MW) toward different genotypes of *M. immunogenum*

In semi-synthetic MWF matrix, the reference genotype showed relatively higher susceptibility to Kathon in comparison with the other genotypes, as indicated by its observed 1 to 2-log reduction for every 1,000 ppm increment in biocide level and a complete inactivation at 5,000 ppm biocide level (Fig. 2a). MJY-4 and MJY-12 showed a relatively slower rate of log reduction in cell count and a complete inactivation at 6,000 and 8000 ppm,

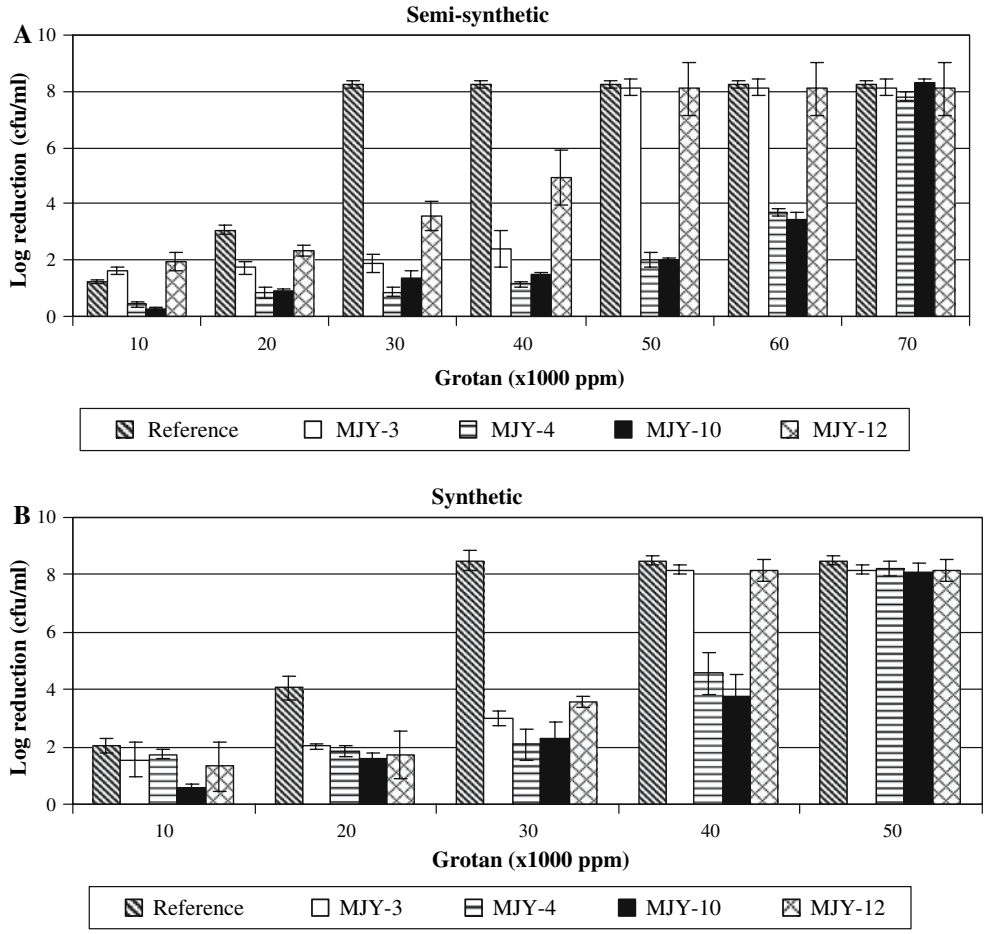

Fig. 1 Determination of the relative efficacy of Grotan biocide toward different genotypes of *M. immunogenum* (reference, MJY-3, MJY-4, MJY-10 and MJY-12) presented as logarithmic reduction in the cell concentration. The efficacy was tested at varying concentrations of the biocide using 60 min contact time. **a** Log_{10} reduction of viable cells in semi-synthetic metalworking fluid (MWF). **b** Log_{10} reduction of viable cells in synthetic MWF

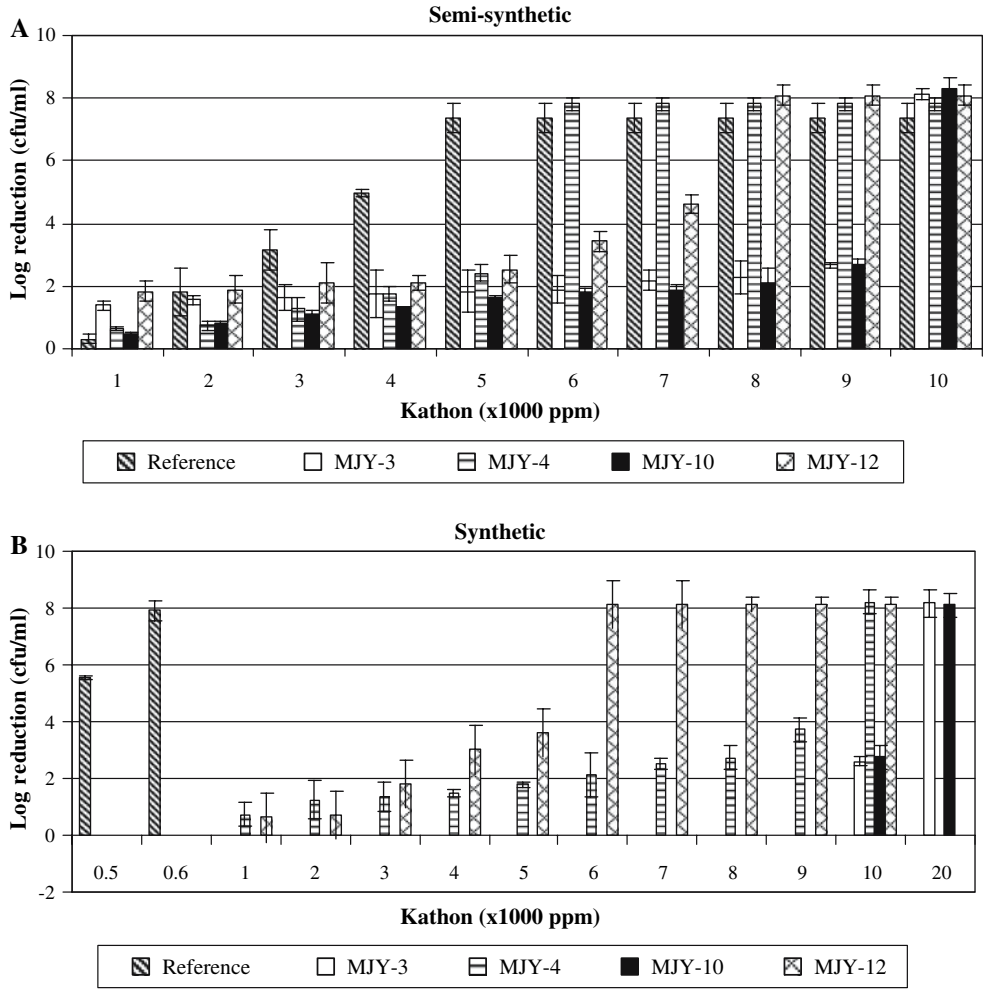

Fig. 2 Determination of the relative efficacy of Kathon biocide toward different genotypes of *M. immunogenum* (reference, MJY-3, MJY-4, MJY-10 and MJY-12) presented as logarithmic reduction in the cell concentration. The efficacy was tested at varying concentrations of the biocide using 60 min contact time. **a** Log_{10} reduction of viable cells in semi-synthetic metalworking fluid (MWF). A common test concentration range (1,000–10,000 ppm) was used for all test genotypes. **b** Log_{10} reduction of viable cells in synthetic MWF. A variable test concentration range was used for different test genotypes, as guided by the observations in **a**. The following concentration ranges were used: 100 ppm increments for the reference genotype, 1,000 ppm increments for MJY-4 and MJY-12 genotypes, and 10,000 ppm increments for MJY-3 and MJY-10 genotypes

respectively. The slowest rate of log reduction was observed for the genotypes MJY-3 and MJY-10, which showed a complete inhibition at as high as 10,000 ppm concentration (Fig. 2a).

The results on synthetic fluid matrix are given in Fig. 2b. Selection of the test concentration range for this part varied with the genotype and was guided by the preceding observations with semi-synthetic fluid. The log-reduction values were determined using 100 ppm increments in biocide concentration for the reference genotype and larger increments for the other genotypes: 1,000 ppm increments for MJY-4 and MJY-12 and 10,000 ppm increments for MJY-3 and MJY-10 (Fig. 2b). The reference genotype showed a significant inactivation (5-log reduction) even at the lowest test concentration (500 ppm) and a complete loss of viability at 600 ppm. In contrast, the other genotypes showed much higher resistance. MJY-12 showed about 1-log reduction for every 1,000 ppm increase in biocide concentration and a complete inactivation at 6,000 ppm. MJY-4 showed a gradual initial reduction in cell number resulting in about a 4-log reduction at 9,000 ppm, and a complete inactivation at 10,000 ppm level. MJY-3 and MJY-10 were the least susceptible showing first detectable inactivation (3-log reduction) at 10,000 ppm biocide level and a complete inactivation at 20,000 ppm (Fig. 2b).

The reference genotype showed the highest Kathon susceptibility in both synthetic and semi-synthetic fluid matrices. Among the isolated genotypes, MJY-4 and MJY-10 showed relatively lower Kathon susceptibility as compared to the other genotypes (Fig. 2). All genotypes except for the

reference strain and MJY-12 showed relatively higher biocidal tolerance in synthetic MWF as compared to semi-synthetic MWF. The general trend in Kathon susceptibility of different test genotypes was as follows: reference genotype > MJY-12 > or < MJY-4 > MJY-3 > MJY-10.

Relative MICs of individual genotypes for the test biocides

Minimum inhibitory concentration values of the two test biocides, Grotan and Kathon, toward the five different genotypes of *M. immunogenum* are given in Table 1. Between the HCHO-releasing biocide Grotan and Isothiazolone biocide Kathon, the latter showed lower MIC values against all genotypes of *M. immunogenum* in both MWF fluid matrices. All genotypes (except the reference strain) showed higher MICs to the HCHO-releasing biocide (Grotan) in SS as compared to S fluid matrix. However, in case of the isothiazolone biocide (Kathon), a better biocidal activity (lower MIC) was observed in SS fluid for majority of the test genotypes (MJY-3, MJY-4 and MJY-10). The MIC values for the reference genotype were 25,000 ppm (S) and 18,000 ppm (SS) for Grotan and 600 ppm (S) and 5,000 ppm (SS) for Kathon. Overall, among the test genotypes, MJY-10 gave the highest MIC values for both Grotan (50,000 and 70,000 ppm) and Kathon (20,000 and 10,000 ppm) in S and SS MWF matrices, respectively (Table 1). Collectively, the results indicated that the isolated genotypes are more resistant than the reference genotype to either biocide in both fluid types.

Discussion

Mycobacteria have been known for their resistance against antimicrobial agents as compared to the relatively more sensitive gram-negative and gram-positive bacteria [13]. The hydrophobic, waxy, and impermeable nature of the mycobacterial cell wall, which contains mycolic acids, arabinogalactan and high molecular weight lipids, has been postulated to be responsible for their antimicrobial resistance [2]. While this general trend on the relative resistance extends to the mycobacteria species associated with metal-working fluids [16], the extent of resistance may vary considering a continual exposure to multiple biocides prevalent in these fluids. However, there is complete lack of specific information on the relative biocidal susceptibility of the actual strains of mycobacteria colonizing these niches.

Table 1 Minimum inhibitory concentrations for the different genotypes of *Mycobacterium immunogenum*: effect of the test biocide and the fluid matrix type

Genotype	Minimum inhibitory concentration (ppm)			
	Grotan		Kathon	
	Synthetic MWF	Semi-synthetic MWF	Synthetic MWF	Semi-synthetic MWF
Reference	25,000	18,000	600	5,000
MJY-3	40,000	50,000	20,000	10,000
MJY-4	50,000	70,000	10,000	6,000
MJY-10	50,000	70,000	20,000	10,000
MJY-12	40,000	50,000	6,000	8,000

The minimum inhibitory concentration value corresponded to the lowest biocide concentration that caused complete growth inhibition (100% inactivation) of *M. immunogenum* at 60 min contact time

Role of biocide type

The results showed that Kathon (Methylisothiazolone) biocide is effective against *M. immunogenum* genotypes at relatively lower concentrations than the HCHO-releasing biocide Grotan when compared in either of the two MWF matrices (synthetic or semi-synthetic). As different biocides have different mechanisms of action, this difference could be the likely basis for the increased effectiveness of Isothiazolone biocide Kathon against the test mycobacteria. The relatively lower effectiveness (in terms of MIC) of HCHO-releasing biocide Grotan, against *M. immunogenum* genotypes, could be ascribed to the impaired surface activity of its released active component (formaldehyde) on mycobacterial cells [4]. The hydrophobic and waxy nature of mycobacterial cell wall discourages such interaction of formaldehyde and thus provides protection against HCHO biocides. On the other hand, Isothiazolone biocides induce the accumulation of toxic free radicals inside the bacterial cells that leads to "biocide-induced autocidal activity". Also, methylisothiazolone interacts with the thiol group in proteins, which is essential for the activity of many respiratory enzymes [7]. Nevertheless, there was certain degree of Kathon resistance observed among the test mycobacteria. One possible reason for this could be the variable mycobacterial detoxification of thiol reactive substances by the production of Mycothiol, a unique thiol comprised of *N*-acetylcysteine amide produced in mycobacteria [10]. Mycothiol, is a thiol compound unique to mycobacteria and actinomycetes but not produced in gram-positive and most of the gram-negative bacteria. Likewise, the mycobacterial resistance to HCHO-biocides could be because mycothiol detoxifies the active component formaldehyde [10]. Resistance of the closely related species *M. chelonae* to glutaraldehyde and other aldehydes has been observed in other studies related to hospital hygiene [19, 20].

Role of matrix type

The results showed that the type of MWF fluid matrix has an impact on biocidal activity of both Grotan and Kathon.

An increased biocidal activity of Grotan was observed in synthetic MWF as compared to semi-synthetic fluid except for the reference genotype. In contrast, Kathon was observed to be more effective in semi-synthetic MWF as compared to synthetic fluid except for the reference genotype and MJY-12 genotype. This is consistent with the reported observation that chemical compatibility between the type of biocide and MWF matrix type is an important factor while choosing the biocide to control microbial contaminants in MWF [12]. Biocide interactions (synergism, antagonism, neutralism) with functional formulation compounds of MWF are an important factor to be considered in controlling MWF contaminants. The observed increased biocide activity of Grotan in synthetic fluid could be due to the synergistic interaction between triazine (active ingredient of Grotan) and ethanolamines (corrosion inhibitor) of the fluid [12].

Genotype differences

The observed differential biocide susceptibility of *M. immunogenum* genotypes could be ascribed to the intrinsic microbial factors such as genetic make up and cell wall permeability or to acquired mechanisms such as efflux systems, overproduction of target, and inactivity or absence of enzyme/metabolic pathway, or to environmental factors including prior repeated exposure to biocides, and incorrect use of biocides [14]. In support of the intrinsic factors, the variable biocide susceptibility of individual genotypes could be encoded by their variable genotypic profiles [6]. As for the role of variable cell wall permeability, modification in cell wall was reported to be responsible for the varied antimicrobial susceptibility of individual strains of the related mycobacterial species *Mycobacterium chelonae* [8]. Mycothiol synthesis is an important function in mycobacteria for protection against oxidative damage (isothiazolone) and inactivation of electrophilic toxins (triazines) generated from certain biocides. Differential expression of the genes (*mshA*, *mshB*, *mshC* and *mshD*) responsible for mycothiol synthesis [10] could underlie the observed variation in biocide susceptibility of genotypes and needs further confirmation. In this context, increased biocide susceptibility had been observed for the mycothiol deficient *Mycobacterium smegmatis* mutants [11]. An important additional factor in differential susceptibility of individual test strains in our study could be the type or level of their prior exposure to various biocides in industrial operations and their adaptive evolution in that environment. *M. immunogenum* genotypes tested in this study were isolated from MWF samples drawn from diverse fluid operations located in different geographical regions of the country. So, different regimes or levels of biocide exposure at different industrial plants could be responsible for the observed differential biocide susceptibility of genotypes in this investigation. Collectively, the intrinsic genetic factors leading to changes in cell wall (causing variable permeability) and mycothiol synthesis and a prior exposure of test organisms to biocides, could be among the likely critical factors responsible for differential biocide susceptibility of different genotypes of *M. immunogenum.*

In conclusion, the study shows differential biocidal susceptibility of the mycobacteria genotypes prevalent in industrial metalworking fluids and emphasizes the need to incorporate these genotypes (strains) in the scheme of development and/or evaluation of the existing and future biocide formulations for control of mycobacterial growth in these occupational niches. Future interests are in the characterization of the genetic and molecular basis of the variable resistance to biocides in these MWF mycobacteria strains. With the likely future availability of genomic sequences, there is high possibility of knowing the strain-specific changes in genetic make up and specific identification of antimicrobial resistance genes in these organisms.

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