CURRENT MICROBIOLOGY Vol. 46 (2003), pp. 28–32
DOI: 10.1007/s00284-002-3796-4

Current Microbiology
An International Journal

Biofilm Formation and Biocide Susceptibility Testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*

Elias Bardouniotis,[1] Howard Ceri,[1,2,3] Merle E. Olson[1,2,3]

[1]Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, Alberta T2N 1N4, Canada
[2]Biofilm Research Group, University of Calgary, 2500 University Drive N.W., Calgary, Alberta T2N 1N4, Canada
[3]Microbiology and Infectious Diseases, University of Calgary, Health Sciences Centre, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada

Received: 15 February 2002 / Accepted: 28 March 2002

Abstract. The ability of non-tuberculous mycobacteria to form biofilms may allow for their increased resistance to currently used biocides in medical and industrial settings. This study examines the biofilm growth of *Mycobacterium fortuitum* and *Mycobacterium marinum*, using the MBEC™ assay system, and compares the susceptibility of planktonic and biofilm cells to commercially available biocides. With scanning electron microscopy, both *M. fortuitum* and *M. marinum* form biofilms that are morphologically distinct. Biocide susceptibility testing suggested that *M. fortuitum* biofilms displayed increased resistance over their planktonic state. This is contrasted with *M. marinum* biofilms, which were generally as or more susceptible over their planktonic state.

Non-tuberculous mycobacteria (NTM), which comprise more than 65 species, are defined as those mycobacteria that are not part of the *Mycobacterium tuberculosis* complex. Infections by NTM are increasing in both industrialized and developing countries, owing in part to an increase in the aging population, immunosuppressive therapy, the emergence of AIDS, and an increase in surveillance [7]. The rapid-growing *M. fortuitum* is an opportunistic NTM that has been identified in several nosocomial infections involving hospital instruments [12], hospital water systems [10], and peritoneal catheters [11]. It has also been detected in samples from drinking water, lakes, ponds, and distilled water [3, 5, 13]. The slow-growing *M. marinum* can act as a zoonoses transmitted directly from fish or through swimming pools and fresh or salt water to humans; moreover, it is known for causing 'fish tuberculosis,' which is a widespread economical problem for those operating fish aquaculture facilities [2].

A biofilm is defined as a consortium of microbes that adhere to either abiotic or biotic surfaces. Upon adherence, the cells begin micro-colony formation and produce extracellular polymer substance (EPS). Biofilms are known to be difficult to eradicate compared with their planktonic (suspension) counterparts [4, 6, 18]. Experimental evidence has shown that various NTM reside within biofilms [1, 8, 9, 16, 17], and this attribute may result in an increase in their recalcitrance towards biocides, resulting in contamination of clinical and industrial settings.

In this study, we characterize biofilm growth, report biocide susceptibility testing, and provide visual evidence of biofilm morphology by using scanning electron microscopy (SEM) of *M. fortuitum* and *M. marinum*.

Correspondence to: M.E. Olson; *email*: molson@ucalgary.ca

Materials and Methods

Bacterial culture and growth media. All experiments were performed with Middlebrook 7H9 broth (MB; Difco) supplemented with ADC enrichment (BBL) and Middlebrook 7H10 agar (MA; Difco) supplemented with OADC enrichment (BBL) and glycerol. *M. fortuitum* 6854 and *M. marinum* 84517 are both clinical isolates that were obtained from the Provincial Laboratory of Alberta (Calgary). Both strains were recovered from polystyrene spheres (Microbank™) maintained at −70°C and were grown by streaking polystyrene spheres over MA. The biofilm inoculum was initiated in broth culture containing 90 ml of MB supplemented with 10 ml ADC enrichment in a shaking incubator (110 revolutions min^{-1}) for 3 or 5 days at 37°C or 28°C for *M. fortuitum* and *M. marinum*, respectively.

Biofilm formation and growth rate. *M. fortuitum* and *M. marinum* biofilms were formed by using the MBEC™ assay system (MBEC™ Biofilm Technologies Ltd., Calgary, Alberta) as previously described

with slight modifications [4]. The MBEC™ device employs a 96-peg lid that is sealed on top and has a ridged trough on the bottom. The top plate lid fits over the bottom trough, which contains the microbial inoculum, and the device is placed on a rocking platform, thereby allowing a shear force to be created forming equivalent biofilms on all 96 pegs. A volume of 25 ml of the 3- or 5-day broth culture was inoculated into the MBEC™ device, which was then incubated on a rocking platform. Biofilm growth curve counts were determined by removing pegs from the peg lids and placing them in 200 μl of MB in a microtiter plate. The pegs were then sonicated with an Aquasonic (model 250HT; VWR Scientific) sonicator on high setting for 5 min, serially diluted, and spot plated on MA to determine colony-forming unit (CFU) counts.

Biocide susceptibility testing. Biocide susceptibility testing was performed as previously described by Bardouniotis et al. [1] with slight modifications. Briefly, working solutions of selected biocides were prepared in sterile distilled water, and serial twofold dilutions were prepared in standard 96-well plates. Biocide testing was performed on day 3 and day 14 for *M. fortuitum* and *M. marinum*, respectively, and all biocide dilutions, were prepared the day of testing. The biofilms were assayed as follows: The MBEC™ peg lids were removed from the rocking inoculum at day 4 for *M. fortuitum* and day 14 for *M. marinum* in order to achieve 1×10^5–1×10^6 CFU/peg. The peg lid was rinsed in 40 ml of 0.9% saline to remove planktonic bacteria and was placed over a 96-well plate in which various biocides were diluted. Plates were incubated for 30 or 120 min at 20°C ambient temperature. Following the challenge incubation, the peg lids were rinsed twice with 0.9% saline for 1 min to remove any residual biocide on the pegs. The peg lids were transferred to another 96-well microtiter plate containing 200 μl of MB recovery media, sonicated for 5 min on a high setting, and 20 μl was spot plated on MA to determine bacterial growth. The planktonics were assayed as follows: the 5- and 7-day inoculums of *M. fortuitum* and *M. marinum* were diluted 1:10, and 10 μl of this was added directly to a 96-well plate containing the prepared biocides. The plates were then challenged for 30 or 120 min at 20°C ambient temperature and serially diluted and spot plated as above. The Minimal Biofilm Eradication Concentration (MBEC) was defined as the minimal concentration of the biocide needed to treat the biofilm for which there is no bacterial growth, and the Minimal Bactericidal Concentration (MBC) was defined as the minimal concentration required to treat planktonic cells where there is no bacterial growth.

Biocide tested. Phenol 1.56% (Sporicidin®, Sporicidin International, Rockville, MD, USA), potassium monopersulfate 21.4% (Virkon®, Dispar division of Vetoquinol Canada Inc., Joliette, Canada), gluteraldehyde 70% (Electron Microscopy Sciences, Ft. Washington, USA), sodium hypochlorite 5.25% (Javex™, Colgate-Palmolive, Canada), hydrogen peroxide 30% (BDH Inc., Toronto, Canada), chlorohexidine acetate 2% (Hibitane®, Ayerst Laboratories, Montreal, Canada), and silver nitrate (Sigma Chemical Co., St. Louis, MO, USA) were used in the study. The biocide concentrations were chosen based on manufacturer's recommendations for disinfection.

Scanning electron microscopy. Pegs were aseptically removed and then fixed in 5% gluteraldehyde/0.1M cacodylate buffer (pH 7.2) for 24 h at 4°C. They were then air dried for 7 days and sputter-coated with gold-palladium prior to being visualized by using a Cambridge S360 scanning electron microscope.

Results

Growth curves. A growth curve for *M. fortuitum* and *M. marinum* was obtained in order to assess their ability to grow as a biofilm and to achieve colony count of 1×10^5–1×10^6 CFU/peg desired to carry out biocide susceptibility testing. The biofilm growth curves of *M. fortuitum* and *M. marinum* are shown in Figs. 1a and 1b, respectively. Maximal growth of *M. fortuitum* of 5.0×10^7 CFU/peg was attained on day 5, whereas *M. marinum* exhibited slower growth, reaching a maximal value of approximately 3×10^5 CFU/peg on day 14. Both species exhibited a sigmoidal growth curve expected with biofilm growth. Biofilm growth of *M. fortuitum* increased 2-log-fold during the first 3 days, with a slight drop-off on day 4 owing to biofilm detachment from the peg; this was followed by a 2.5-log-fold increase at day 5. The growth kinetics of *M. marinum* were similar to those seen with *M. fortuitum* with the exception of an initial 2-log increase seen at day 1; this was followed by

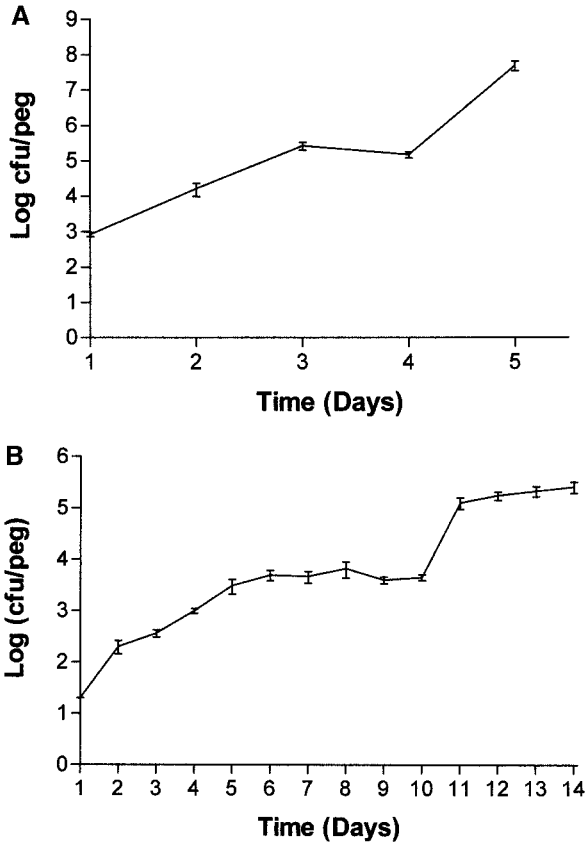

Fig. 1. (A) Growth curve of *M. fortuitum* 6854 in MB supplemented with ADC enrichment by using the MBEC™ assay system. Bacterial counts were determined by removing pegs from the peg lid as previously described in Materials and Methods. Bars represent standard error (S.E.). The solid line denotes CFU average for 3 pegs in one trial. (B) Growth curve of *M. marinum* 84517 in MB supplemented with ADC enrichment by using the MBEC™ assay system. Bacterial counts were determined by removing pegs from the peg lid as previously described in Materials and Methods. Bars represent standard error (S.E.). The solid line denotes CFU average for 3 pegs in one trial.

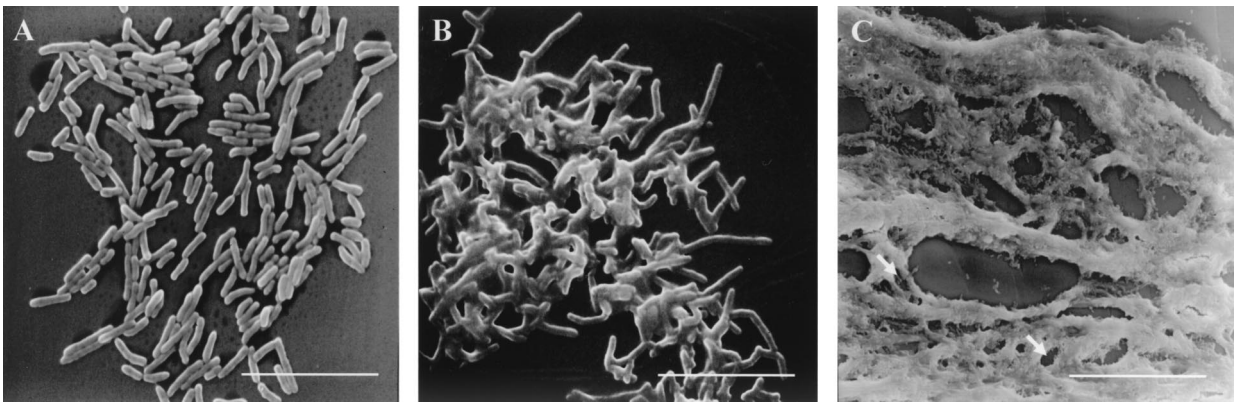

Fig. 2. SEM of *M. fortuitum* 6854 growing on a peg in MB supplemented with ADC enrichment after day 1 (A), day 3 (B), and day 6 (C). Initial adhesion can be seen in A, and different filamentous strands with visible EPS are shown in B. Shown in C is extensive biofilm with channels (arrows). (Scale bars = 5 μm for A and B, and 50 μm for C.)

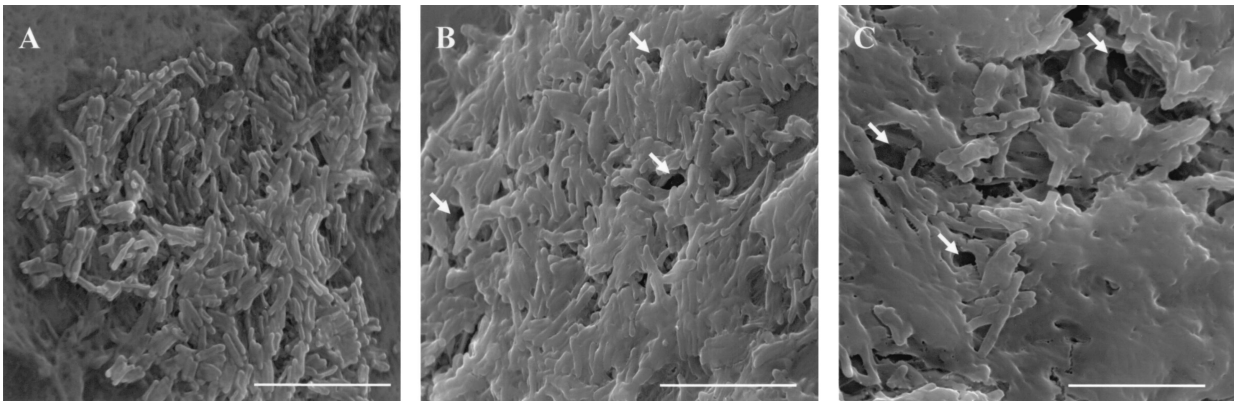

Fig. 3. SEM of *M. marinum* 84517 growing on a peg in MB with ADC enrichment after day 8 (A), day 11 (B), and day (14). Micro-colony formation and extensive EPS can be seen in A and B. Shown in B and C is a biofilm with channels (arrows). (Scale bars = 5 μm.)

a steady increase up to day 9, where a small decline was seen followed by a sharp increase and levelling off by day 14.

SEM. SEM analysis was carried out to in order to observe the biofilm morphology of *M. fortuitum* and *M. marinum* during different times of biofilm growth. As per Fig. 2A, on day 1, short rods are seen adhering to the surface of the peg, followed by the formation of different-sized filamentous strands and the formation of a thick EPS on day 3 (Fig. 2B). As per Fig. 3A, micro-colonies of *M. marinum* composed of EPS are demonstrated, followed by an increase in EPS on day 11 (Fig. 3B). On day 5, *M. fortuitum* (Fig. 2C) and day 14 *M. marinum* (Fig. 3C) both demonstrated a thick-layered mature biofilm composed of channels.

Biocide susceptibility testing. Biofilm and planktonic bacteria were exposed to various biocides for 30 and 120 min. The data corresponding to biocide susceptibility testing at 30 and 120 min for *M. fortuitum* and *M. marinum* is shown in Tables 1 and 2, respectively. All biocide concentrations are reported in parts per million (ppm) converted from their original percentage values. The MBEC and the MBC values correspond to biofilm and planktonic bacteria, respectively. All tests were highly reproducible, with values being the same or at most being within a one-dilution of difference. A greater concentration of biocide was required to eliminate *M. fortuitum* biofilms compared with the planktonic bacteria (Table 1). This was evidenced by the higher MBEC values compared with the MBC values, with the exception of Sporicidin® and hydrogen peroxide at 30 min. The same trend was seen at 120 min with the exception of hydrogen peroxide, Sporicidin,® and Virkon®. A general time dependence of susceptibility was also observed even at periods of exposure as long as the 30 and 120 min challenge incubations, with the exception of the MBC values with bleach and gluteraldehyde and the MBEC of

Table 1. Comparison of MBEC and MBC in parts per million (ppm) for various biocides after a 30- and 120-min challenge with *M. fortuitum* 6854[a]

	30 min		120 min	
Biocide	MBC ppm	MBEC ppm	MBC ppm	MBEC ppm
Bleach	53	2000	53	500
Hydrogen peroxide	>75,000	>75,000	75,000	75,000
Hibitane®	88	>7500	44	>7500
Sporicidin®	15,600	13,000	7800	7800
Gluteraldehyde	2500	2875	2500	563
Silver nitrate	26	313	<10	234
Virkon®	8300	40,000	1250	1250
Control[b]	—	—	—	—

[a] Values are a mean of n = 3.
[b] Sterile water was used as a biocide control.

Table 2. Comparison of MBEC and MBC in parts per million (ppm) for various biocides after a 30- and 120-min challenge with *M. marinum* 84517[a]

	30 min		120 min	
Biocide	MBC ppm	MBEC ppm	MBC ppm	MBEC ppm
30 min			120min	
Bleach	<13	26	<13	26
Hydrogen peroxide	9375	9375	4687	1171
Hibitane®	>325	650	>325	163
Sporicidin®	7800	7800	3900	975
Gluteraldehyde	1250	2500	1250	313
Silver nitrate	323	1352	323	676
Virkon®	10000	2500	10000	2500
Control[b]	—	—	—	—

[a] See footnote [a] of Table 1.
[b] See footnote [b] of Table 1.

Hibitane®. Generally, the biofilm bacteria showed greater resistance to the various biocides compared with their planktonic forms.

No increased resistance of *M. marinum* biofilms over their planktonic state was generally observed (Table 2). However, at 30 min *M. marinum* biofilms displayed an increased resistance over their planktonic state with bleach, Hibitane®, gluteraldehyde, and silver nitrate, with MBEC values greater than those of the MBC. At 120 min, the planktonic cultures were more resistant than biofilm cultures to a given biocide with the exception of bleach and silver. Time-dependent susceptibility was generally observed for biofilm treatment with the exception of bleach and Virkon®, which maintained identical MBEC values at both 30 and 120 min. The planktonic forms generally displayed similar MBC values at 30 and 120 min, with the exception of treatment with hydrogen peroxide and Sporicidin®.

Discussion

Various NTM have been shown to exist in monospecies biofilms; however, we know little as to the differences in biocide suscptibility between most species of NTMs as biofilms and planktonic cultures. We employed the MBEC™ assay system, which we described previously in a study of biocide susceptibility of *M. phlei* as a model system to study the susceptibility of important pathogenic species *M. fortuitum* and *M. marinum* [1, 4]. The growth kinetics of *M. fortuitum* and *M. marinum* demonstrate that both rapid- and slow-growing NTM can form biofilms. Furthermore, growth occurred in the expected sigmoidal fashion, which is consistent with observed biofilm formation by NTMs in other biofilm formation studies [1, 8]. *M. fortuitum* and *M. marinum* demonstrated different biofilm morphologies under SEM. *M. fortuitum* demonstrated aggregation into heterogeneous filamentous strands with visible EPS, as has previously been shown [8], whereas *M. marinum* appeared to form more classical micro-colony formation, as was reported for *M. phlei* [1]. In addition, we are able to demonstrate channels, in both species, a hallmark of biofilm formation. These channelling systems are believed to be responsible for cycling nutrients and wastes into and out of the biofilms and have been observed with mycobacterial biofilms elsewhere [8, 9].

It has been stated previously that many biocides in use today are efficacious against planktonics but not biofilm microbes [4, 6]. The MBEC™ assay system described previously [1, 4] was used to evaluate the biocide susceptibilities of the pathogenic mycobacteria, *M. fortuitum* and *M. marinum.* The attributes responsible for increased resistance of biofilms has been previously discussed [18], and the ability of *M. fortuitum* to form biofilms lends support to its increasing isolation. Moreover, Uttley et al. [19] hypothesize that the ability of *M. fortuitum* to reside within biofilms on endoscopes may allow for an increased resistance to current biocides, thereby affecting its removal. In this study, we support this claim as evidenced by the increased resistance of *M. fortuitum* biofilms over their planktonic forms to the various biocides tested. Although *M. marinum* was generally resistant to killing by the various biocides at 30 min, at 120 min the MBC values were similar to or higher than the MBEC values, indicating that the planktonics were as or more resistant than the biofilms. This raises the issue that, although the biofilms have been repeatedly shown to have a decreased susceptibility to antimicrobial agents, this may not always be the case.

This would suggest that each biofilm organism must be considered on its own as to its susceptibility of antibiotics and biocides. NTM are emerging pathogens, as evidenced by their increasing isolation from patients and hospital environments. They have been found to contaminate industrial and medical settings, likely owing to the inability of current biocides to eradicate them. NTM have been recovered from hospital water and hospital instruments, resulting in nosocomial infections, suggesting that residence within biofilms allows for their proliferation. Stoodley et al. [9] have further demonstrated the ability of *M. fortuitum* and *M. chelonae* to form biofilms on silastic rubber, which is commonly found on medical instruments and high-density polyethylene present in water distribution systems. In addition, finding NTM concentrations 400 times greater in dental spray and cooling water than that of normal drinking water is highly suggestive of residence within biofilms [17]. NTM biofilms have also been found to exist in nature, and an elegant model has been elucidated that demonstrates the mechanism of mycobacterial biofilm formation [14].

Although this work was performed in vitro, factors mentioned previously may influence the ability of biocide agents to eradicate mycobacterial biofilms [15]. It is, therefore, important that steps be taken to ensure that personnel are properly educated with regards to the proper maintenance of disinfection processes in medical and industrial settings. To date there is no universal test method to evaluate biocide susceptibilities of mycobacteria, although many studies have been published [15]. The ability of mycobacteria to reside within biofilms may result in an increased resistance to commonly used antimicrobials. There is a trend of variability in the susceptibility of *Mycobacterium* species to various biocides, to fully evaluate the susceptibility of biofilms, one needs to evaluate different strains and species. Furthermore, the data suggest that each isolate needs to be tested separately for each biocide application. The various hypotheses that exist with respect to the differences between biofilm and suspension microbes to antimicrobial treatments is still an area of great debate and requires further examination; however, it is clear that biofilms must be looked at in a way different from that for planktonic isolates.

ACKNOWLEDGMENTS

This research was supported by grants by the National Sciences and Engineering Research Council of Canada (NSERC) given to M. E. Olson and H. Ceri and by funds provided by the Westaim/ASRA Chair for Biofilm Research. We would like to thank Liz Middlemis for help with the scanning electron microscopy and Wayne Jansen for his technical assistance.

Literature Cited

1. Bardouniotis E, Huddleston W, Ceri H, Olson ME (2001) Characterization of biofilm growth and biocide susceptibility testing of *Mycobacterium phlei* using the MBEC™ assay system. FEMS Microbiol Lett 203:263–267
2. Bercovier H, Vincent V (2001) Mycobacterial infections in domestic and wild animals due to *Mycobacterium marinum, M. fortuitum, M. chelonae, M. porcinum, M. farcinogens, M. smegmatis, M. scrofulaceum, M. xenopi, M. kansasii, M. simiae* and *M. genavense*. Rev Sci Tech OIE (Off Int Epizoot) 20:265–290
3. Carson LA, Peterson NJ, Favero MS, Aguero SM (1978) Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. Appl Environ Microbiol 36:839–846
4. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A (1999) The Calgary biofilm device: new technology for the rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 37:1771–1776
5. Collins CH, Grange JM, Yates MD (1984) Mycobacteria in water: a review. J Appl Bacteriol 57:193–211
6. Costerton JW (1999) Introduction to biofilm. Int J Antimicrob Agents 11:217–221
7. Falkinham JO III, (1996) Epidemiology of infection by non-tuberculous mycobacteria. Clin Microbiol Rev 9:177–215
8. Hall-Stoodley L, Lappin-Scott H (1998) Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum*. FEMS Microbiol Lett 168:77–84
9. Hall-Stoodley L, Keevil CW, Lappin-Scott HM (1999) *Mycobacterium fortuitum* and *Myobacterium chelonae* biofilm formation under high and low nutrient conditions. J Appl Microbiol 85:60S–69S
10. Kauppinen J, Nousiainen T, Jantunen E, Mattila R, Katila ML (1999) Hospital water supply as a source of disseminated *Mycobacterium fortuitum* infection in a leukemia patient. Infect Control Hosp Epidemiol 20:343–345
11. Kawamoto S, Otani K, Kawaguchi Y, Hosoya T (1999) *Mycobacterium fortuitum* peritonitis associated with CAPD: diagnosis by a molecular biology technique. Infect Control Hosp Epidemiol 19: 592–593
12. Kressel AB, Kidd F (2001) Pseudo-outbreak of *Mycobacterium chelonae* and *Methylobacterium mesophilicum* caused by contamination of an automated endoscopy washer. Infect Control Hosp Epidemiol 22:414–418
13. Papapetropoulo M, Tsintzou A, Vantarakis A (1997) Environmental mycobacteria in bottled table waters in Greece. Can J Microbiol 43:343–345
14. Recht J, Kotler R (2001) Gycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. J Bacteriol 183:5718–5724
15. Russell AD (1996) Activity of biocides against mycobacteria. J Appl Bacteriol 81:87S–101S
16. Schulze-Röbbecke R, Janning B, Fischeder R (1992) Occurrence of mycobacteria in biofilm samples. Tubercle Lung Dis 73:141–144
17. Schulze-Röbbecke R, Feldmann C, Fischeder R, Janning B, Exner M, Wahl G (1995) Dental units: an environmental study of sources of potentially pathogenic mycobacteria. Tubercle Lung Dis 76: 318–323
18. Thien-Fah CM, O'Toole GA (2001) Mechanism of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34–39
19. Uttley AHC, Simpson RA (1994) Audit of bronchoscope disinfection: a survey of procedures in England and Wales and incidents of mycobacterial contamination. J Hosp Infect 26:301–308