Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum*

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Abstract

Rapidly growing mycobacteria (RGM) are found in soil and diverse aquatic environments. Two species, *Mycobacterium fortuitum* and *Mycobacterium chelonae*, are associated with disease and are difficult to eradicate. Biofilm formation may be a contributing factor to their mode of transmission and their resistance to antimicrobial agents. We investigated the ability of the RGM species *M. fortuitum* to colonise surfaces using a modified Robbins device. *M. fortuitum* formed dense biofilms within 48 h. The high numbers of sessile organisms recovered and the swiftness of colonisation suggest that *M. fortuitum* readily forms biofilms. These results suggest a novel mechanism for mycobacteria in evading antimicrobial treatment and also indicate that biofilms should be considered possible sites for mycobacterial contamination. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Mycobacteria other than *Mycobacterium tuberculosis* are known as non-tuberculous mycobacteria. One group, the rapidly growing mycobacteria (RGM), are chiefly saprophytic, living in soil or aquatic habitats. However, two species, *Mycobacterium fortuitum* and *Mycobacterium chelonae*, are opportunistic pathogens affecting people with underlying immune dysfunction or chronic disease [1–6]. RGM are also associated with post-surgical infections or medically invasive procedures. Several outbreaks of *M. fortuitum* and *M. chelonae* have been linked to a hospital or clinical source [6–11].

Certain features of RGM growth and epidemiology suggest that they may form biofilms. First, they are commonly isolated from aquatic habitats including water distribution systems and tap water [1,12]. Secondly, several clinical outbreaks have been associated with medical devices that were stored or cleaned in distilled or tap water [7–11]. Thirdly, RGM are associated with resistance to both disinfectants and antibiotics [3,4,13], an attribute consistently seen in biofilm forming organisms [14]. Finally, there is some direct evidence that mycobacteria can exist in adherent or slimy sediments within water distribution systems. Schultze-Röbbecke et al. recovered both slow and rapidly growing mycobacteria in 45 of 50 biofilm samples taken from municipal or domestic water supplies in Germany and France [15,16], but these experiments

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were conducted over several months or years and did not include time periods of less than 4 weeks. Vess et al. also found that *M. chelonae* colonised PVC pipes, but colonisation was again not examined before 8 weeks or under flow conditions [17].

The ability of pathogenic RGM to form biofilms has important implications regarding the epidemiology of these organisms, their ability to survive in the environment and their resistance to biocides. The objective of this study was to assess whether the RGM species *M. fortuitum* formed biofilms in the short term under flow conditions. A modified Robbins device (MRD) was used to grow and sample biofilms. Silastic rubber was chosen for the test surface because it is commonly used in the medical environment [18].

2. Materials and methods

2.1. Culture conditions

M. fortuitum (isolate 10384 from the National Collections of Industrial and Marine Bacteria) was recovered from frozen stock cultures on solid medium (Oxoid blood agar base No. 2 without the addition of blood). For MRD experiments, *M. fortuitum* was transferred to 100 ml of Middlebrook 7H9 broth supplemented with 10% (w/v) OADC enrichment medium (Difco) and grown for 4 days in an orbital shaking incubator (110 revolutions min^{-1}). Exponentially-growing batch cultures (2–3 days old) were sub-cultured twice prior to inoculation into the MRD system. All cultures were grown at 37°C.

2.2. MRD experiments

The MRD is a polycarbonate flow cell with a rectangular lumen lined with sampling studs that can be aseptically removed for analysis. It is used as an in situ sampling device for assessing biofilm growth in medical, environmental and industrial systems (described fully in [19]. Silastic rubber disks were glued in place onto the studs (Fig. 1). A 3% (v/v) inoculum of a 72-h *M. fortuitum* batch culture was introduced into a culture reservoir 18 h before the initiation of flow. It was previously determined that after 18 h the culture was in early exponential phase with an opti-
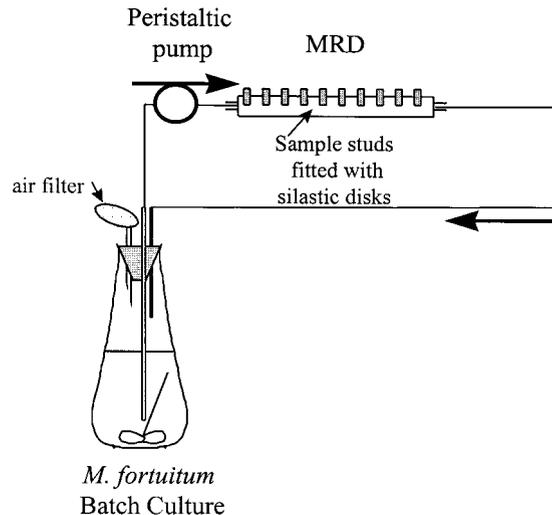

Fig. 1. The MRD and experimental apparatus used for studying *M. fortuitum* biofilm formation. Studs can be aseptically removed for analysis of colonised bacteria. The batch culture was stirred and maintained at 37°C.

cal density (540 nm) of 0.555 ± 0.015. The batch culture was recirculated through the MRD at a continuous flow rate of 54 ml h^{-1} (flow velocity $= 8.33 \times 10^{-4}$ m s^{-1}; Reynolds number $= 3.9$). The experiment was maintained at 37°C and repeated 3 times.

2.3. Sampling protocol and microscopy

2.3.1. Planktonic cells

Planktonic samples were obtained by aseptically removing 50–100 µl of culture medium from the lumen of the MRD via a stud port. Viable cells were assessed at each sampling time by plating triplicate samples of the appropriate dilution onto agar plates. The plates were incubated at 37°C for 48–72 h in candle jars to provide an enhanced CO_2 atmosphere.

2.3.2. Biofilm cells

Samples of attached cells were taken at 2, 6, 12, 24, 36 and 48 h by removing either 2 (experiment 1) or 3 studs (experiments 2 and 3). Studs were randomly selected at each time point to avoid variability in hydrodynamics and nutrient availability along the length of the MRD. Non-adherent cells were washed from the disk by rinsing with 10 ml of sterile 25% Ringers solution. Adherent cells were removed by

scraping as described by Brading et al. [20]. Viable cells were expressed as colony-forming units (CFU) ml^{-1} for planktonic cells or CFU cm^{-2} for attached cells.

The growth rates and doubling times for both planktonic and attached cells were calculated from average cell counts obtained from duplicate (experiment 1) or triplicate (experiments 2 and 3) studs at each sample time using linear regression analysis (Quattro Pro 6.0). Doubling times are reported with the associated r^2 coefficient.

To observe attached *M. fortuitum* cells directly, silastic disks at each time point from experiment 1 were examined microscopically. The disks were removed from the stud and rinsed to dislodge non-adherent cells and fixed in 5% glutaraldehyde/0.1 M cacodylate (pH 7.4) overnight at 4°C. The fixed samples were rinsed in cacodylate buffer and dehydrated using an ethanol series (30, 50, 70, 100%), air dried and examined under an Olympus BH2 micro-
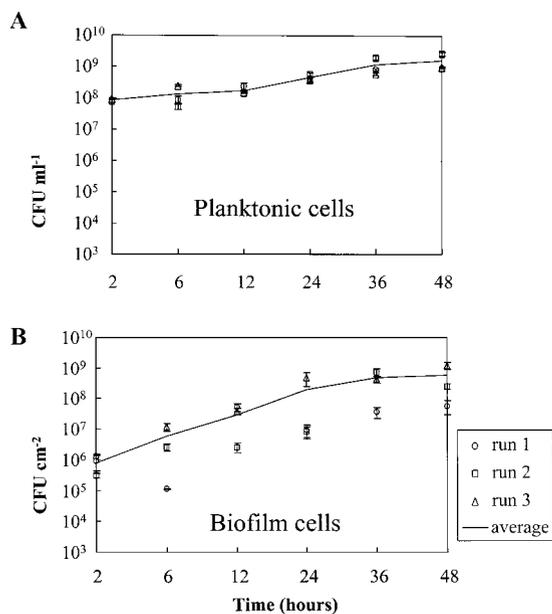
Fig. 2. *M. fortuitum* biofilm formation on silastic rubber. Viable cells (Log$_{10}$) of planktonic CFU ml^{-1} (A) and attached CFU cm^{-2} (B) during a 48-h period. Averages for viable cell counts for each experiment were obtained from either 2 (run 1) or 3 (runs 2–3) studs plated in duplicate (run 1) or triplicate (runs 2–3). Error bars represent 1 standard error (S.E.). The average of all 3 experimental runs is denoted by the solid line (number of studs = 8) and shows an increasing trend in *M. fortuitum* colonisation.
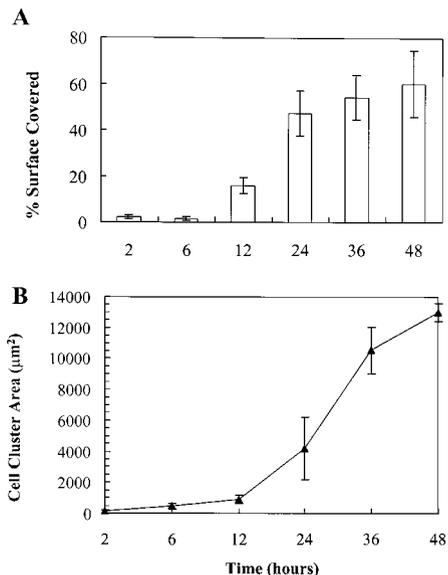
Fig. 3. Accumulation of *M. fortuitum* biofilm (from run 1) as measured by surface percentage cover on silastic disks (A) and average area of individual *M. fortuitum* cell clusters (B). Biofilm accumulation progressed in a sigmoidal fashion. Bars represent 1 S.E.

scope for transmitted light microscopy and image analysis. For scanning electron microscopy (SEM) the dehydrated disks were sputter-coated with a gold-palladium mixture to reduce charge artefacts and visualised using a Cambridge Stereoscan 100 (Leica, Cambridge, UK) with an acceleration voltage of 25 kV.

2.4. Image analysis

To assess surface coverage, a COHU 4612-5000 camera (Cohu, San Diego, CA, USA) connected to a Scion VG-5 PCI framestore board (Scion, Frederick, MD, USA) was used to capture images of biofilms. A threshold was applied so that the cell clusters were black and the surrounding voids white. The relative surface coverage of the biofilm was the proportion of black to the total area [21]. Final surface cover was determined by using the mean of 5 microscopic fields. The area of individual cell clusters was determined by manually outlining the perimeters of the cell clusters. All measurements were calibrated against a 1-mm graticule with 10-μm divisions (Ref. #CS990, Graticules, Tonbridge, Kent, UK).
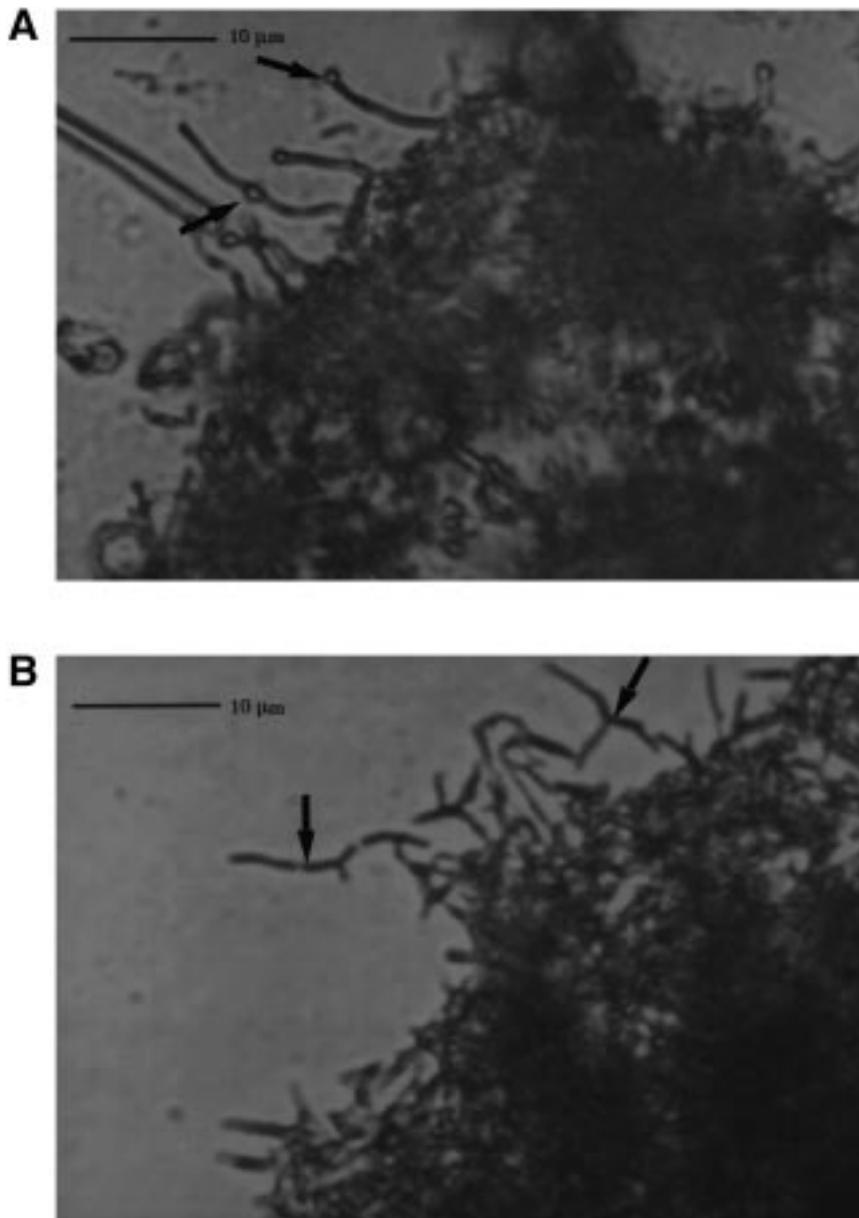
Fig. 4. Phase contrast microscopy showing heterogeneous mycobacterial cell morphology at 2 h (A) and 48 h (B). Scale bars = 10 μm. After 2 h ovoid, light refractive structures (indicated by arrows) were observed on several cells. After 48 h filamentous branching rods (indicated by arrows) were prevalent. The mean length of an individual bacillus was 5.6 ± 0.8 μm (*n* = 12) after 2 h. After 48 h the mean length was 5.0 ± 1.2 μm (*n* = 9). The mean cell width was 0.8 ± 0.05 μm (*n* = 33).
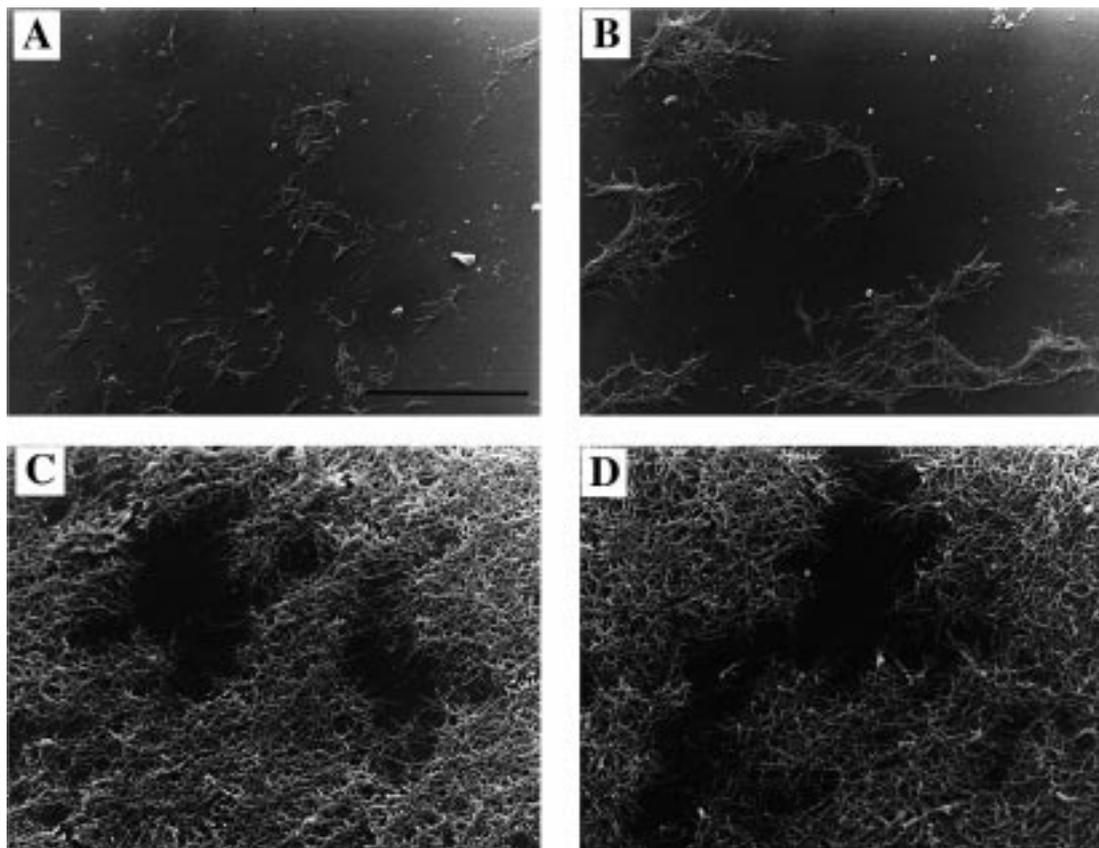
Fig. 5. SEM characterisation of *M. fortuitum* biofilm on silastic rubber after (A) 2 h; (B) 12 h; (C) 24 h; and (D) 48 h. The cell clusters grew larger during the experiment. Disks harvested after 24 h showed cells clusters interspersed with voids in which there were few attached cells (C) and (D). (Scale bars = 50 μm.)

3. Results

3.1. Viable cell enumeration of planktonic and biofilm cells

The average viable planktonic cell population increased from $8.5 \times 10^7 \pm 4.8 \times 10^6$ (mean ± S.E., $n = 6$) CFU ml^{-1} 2 h after flow began to $1.6 \times 10^9 \pm 3.4 \times 10^8$ ($n = 8$) CFU ml^{-1} after 48 h (Fig. 2A). The doubling time for planktonic cells between 2 and 36 h was 8.7 h ($r^2 = 0.99$, $n = 5$). The average viable attached cell population increased exponentially from $7.9 \times 10^5 \pm 1.5 \times 10^5$ ($n = 19$) cells cm^{-2} after 2 h to $5.9 \times 10^8 \pm 1.6 \times 10^8$ ($n = 22$) cells cm^{-2} after 48 h (Fig. 2B). The doubling time for attached cells, 4.4 h ($r^2 = 0.92$, $n = 5$), was significantly different ($P > 0.01$, based on the standard error of the X coefficient) than for planktonic cells.

3.2. Image analysis

Transmitted light microscopy and image analysis were also used to observe the pattern of surface coverage of *M. fortuitum* directly. Fig. 3A shows that *M. fortuitum* surface coverage increased gradually for 48 h. The average area of individual cell clusters also increased (Fig. 3B). The biofilm accumulated sigmoidally with the greatest increase in surface colonisation occurring between 12 and 36 h. The observed area of cell clusters followed a similar sigmoidal pattern (Fig. 3B). After 48 h, $60.3 \pm 14.3\%$ ($n = 5$) of the silastic disk was covered with attached *M. fortuitum* (Fig. 3A).
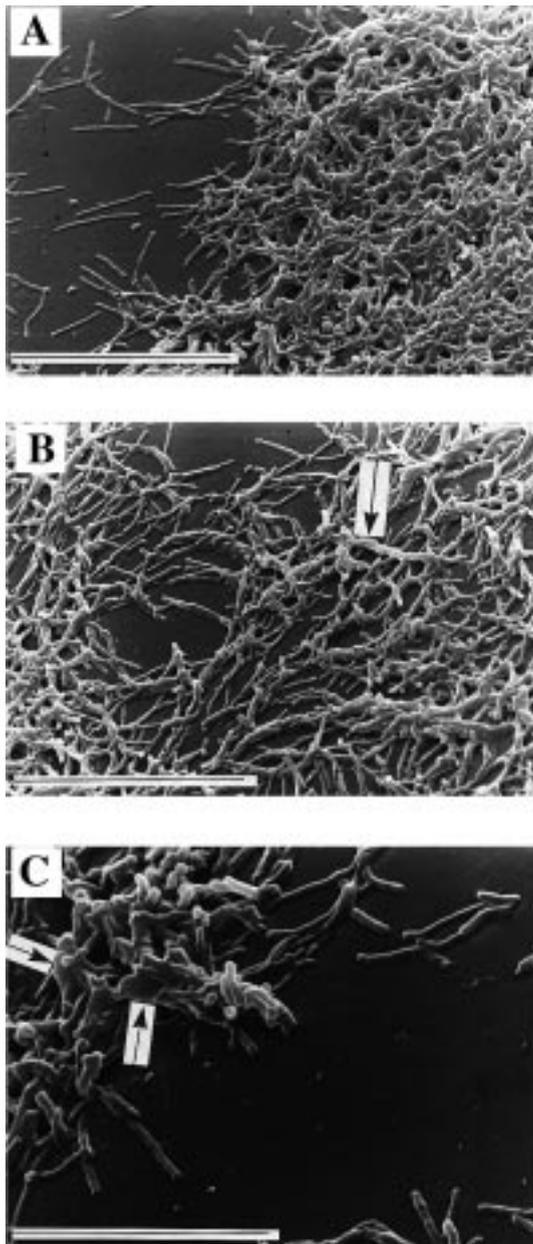
Fig. 6. SEM of *M. fortuitum* biofilm after 24 h (A) and 48 h (B) and (C). Mycobacterial cell heterogeneity can be seen in A and B. Some cells showed a cord-like pattern of aggregation (see arrow in panel B). Possible extracellular material is indicated by arrows in panel C. (Scale bars = 20 μm (A–B) and 10 μm for C.)

Direct microscopy of the biofilm revealed evidence of heterogeneous cell morphology. After 2 h rods that showed ovoid, refractive structures were ob-

served (Fig. 4A). After 48 h branching rods were observed along with straight or curved rods (Fig. 4B).

3.3. SEM

Scanning electron microscopy (SEM) corroborated colonisation trends observed from viable cell counts and image analysis data. Several small cell clusters were evident after 2 h (Fig. 5A). By 12 h cell clusters were larger and showed dense aggregates of cells (Fig. 5B). After 24 h the biofilm was composed of large cell clusters separated by voids (Fig. 5C and D). Higher magnification micrographs indicated that cell clusters exhibited heterogeneous morphology with a mycelial-like texture (Fig. 6A). This morphology was also observed with light microscopy (Fig. 4A and B). Material that resembled extracellular matrix was also present (Fig. 6C). Pleiomorphic cell structures were evident at higher magnification revealing bacilli of different sizes ranging from short curved rods to longer branching rods (Fig. 6A). Cells also aggregated into cord-like strands (Fig. 6A and B).

4. Discussion

Biofilms have been defined as 'matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces' [22]. Using this definition *M. fortuitum* formed biofilms on silastic rubber disks under flow conditions. Both viable cell count data and microscopic data indicated a gradual increase in attached *M. fortuitum* cells (Figs. 2, 3 and 5). Accumulation occurred in a sigmoidal pattern that is generally observed with biofilm formation [23]. The doubling time for attached *M. fortuitum* cells was approximately half that of planktonic cells. This probably reflects that surface accumulation is the sum of both the attachment of planktonic cells and the growth of attached cells, while accumulation in the liquid phase is probably due primarily to growth alone [23]. The faster doubling time exhibited by biofilm cells could also reflect physiological differences between planktonic and attached populations [22].

Both SEM and image analysis data showed a

gradual increase in cell aggregates. These methods allowed fixed, attached cells to be viewed without disrupting the biofilm. Image analysis also allowed quantitative assessment of the non-disrupted mycobacterial biofilm (Fig. 3). Cell clusters were present on surfaces after 2 h of flow through the MRD. After 24 h some areas of the surface were colonised extensively, with dense cell clusters separated by voids that contained few or no cells (Fig. 5C and D, and Fig. 6A). This morphology is consistent with other biofilm structures that exhibited interstitial voids or channels separating cell aggregates [24].

There was some evidence that the *M. fortuitum* biofilm contained an extracellular matrix (Fig. 6C). This material is frequently correlated with adhesion and protection in biofilms [22]. Extracellular material was also observed by Vess et al. who showed *M. chelonae* cells surrounded by an amorphous material after 8 weeks in non-flowing water and postulated that extracellular material may be a principal factor in the survival of water microorganisms after treatment with germicides [17]. Extracellular material has also been observed on a catheter surface colonised by *M. chelonae* [6]. It is also possible that adhesion is primarily due to the hydrophobic properties of the mycobacterial cell wall.

It is significant that the presence of other organisms was not required for colonisation by *M. fortuitum* to occur. This observation suggests that *M. fortuitum* may be a primary colonising organism. Shultze-Robbecke et al. also postulated that mycobacteria are involved in primary colonisation and state that the hydrophobicity of the mycobacterial cell wall makes these organisms 'adhesion specialists of the aquatic environment' [16]. The extent to which *M. fortuitum* formed a biofilm and the alacrity with which it formed suggests that this organism may readily produce biofilms in industrial, medical and natural environments.

RGM have been implicated in a variety of clinically-acquired infections including several outbreaks following cardiac surgery [10]. Wallace et al. found that one *M. fortuitum* isolate was linked to a hospital water supply where the organism was recovered from incoming municipal water mains. One explanation for these outbreaks is that *M. fortuitum* could reside within biofilms. Biofilm reservoirs could explain both the irregularity of outbreaks and the difficulty in identifying a source of the infection, since water samples often fail to include specimens that are adherent to tubing or pipe materials. Based upon the results of the present and previous studies, biofilms should be considered as possible reservoirs for the survival of opportunistic mycobacteria in aquatic environments.

Our results add to the limited body of literature on mycobacterial biofilms by showing that *M. fortuitum* formed biofilms under flow conditions without the presence of other organisms. Biofilms represent a favourable microenvironment for many bacteria. Organisation within a biofilm can provide optimal nutrients and reduced competition from other microbial species. This organisation can also protect bacteria from predation and damaging substances in the environment, including chemical disinfectants and antibiotics [14].

In general, it has been assumed that the resistance of mycobacteria to antibacterial compounds such as disinfectants and antibiotics has been due to three fundamental characteristics: (i) the unique structure of the mycobacterial cell wall as a permeation barrier; (ii) the ability of certain pathogenic mycobacteria to survive within phagocytic cells; and (iii) the rapid mutation of target molecules [25–27]. Our results suggest that a fourth possibility should be considered, at least with environmental mycobacterial species. Mycobacterial resistance to germicides might be in part due to the ability of these organisms to exist in biofilms.

Acknowledgments

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