Growth in catheter biofilms and antibiotic resistance of *Mycobacterium avium*

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Cells of *Mycobacterium avium* strain A5 adhered to plasticized polyvinyl chloride catheter tubing and grew at low nutrient concentration, consistent with reports of catheter-associated *M. avium* infection. Starting with initial cell densities of 1–2 x 10^6 c.f.u. ml^{-1}, biofilms of approximately 350 c.f.u. cm^{-2} formed within 24 h at room temperature. Growth rates of cells in biofilms were exponential and equal to 2.45 days doubling time. Rates were exponential for 1–2 weeks incubation and reached cell densities of 6.5 x 10^6 c.f.u. cm^{-2} by 4 weeks. Cells grown in catheter biofilms were significantly more resistant to clarithromycin and rifamycin than cells grown in suspension.

INTRODUCTION

Catheter-associated infections have been reported for *Mycobacterium avium* (Schelonka et al., 1994), *Mycobacterium neoaurum* (Davison et al., 1988; George & Schlessinger, 1999; Holland et al., 1994; Woo et al., 2000), the *M. neoaurum*-like species *Mycochristium laticolis* (Kiska et al., 2004), *Mycobacterium fortuitum* (Flynn et al., 1988; Raad et al., 1991) and *Mycobacterium chelonae* (Flynn et al., 1988; Raad et al., 1991). These reports all establish that either slowly or rapidly growing mycobacteria isolated from catheters should not be dismissed as laboratory contaminants, but that catheter-associated infection should be considered.

As part of our continuing studies of the ecology of *M. avium* and how habitats occupied by this environmental opportunistic pathogen result in human infection, we have investigated the role of biofilms in *M. avium* persistence in drinking water systems (Steed & Falkinham, 2006). Measurement of numbers of *M. avium* in drinking water systems throughout the world have shown that the majority of *M. avium* cells are in biofilms on pipe surfaces and low numbers are recovered from bulk water (Falkinham et al., 2001; Torvinen et al., 2004). Other mycobacteria, specifically *Mycobacterium kansasi* (Schulze-Röbbecke & Fischeder, 1989), *M. chelonae* (Schulze-Röbbecke et al., 1992), *M. fortuitum* (Hall-Stoodley & Lappin-Scott, 1998) and *Mycobacterium phlei* (Bardouniotis et al., 2001) also form biofilms on surfaces, including high density polyethylene and silastic rubber. It is likely that the high cell surface hydrophobicity of mycobacteria (Van Oss et al., 1975) contributes to biofilm formation. Such a predilection for attachment would also lead to colonization of catheter surfaces.

Counts of mycobacteria growing on surfaces have not been reported in published accounts of mycobacterial catheter-associated infections. Further, there have been no published data on attachment, growth and biofilm formation in catheter tubing by mycobacteria. Based on the observation that cells of other pathogenic micro-organisms grown in biofilms are more resistant to antimicrobial agents (Bardouniotis et al., 2001, 2003; Donlon, 2001; Nickel et al., 1985), it is likely that biofilm-grown cells of *M. avium* in catheters are relatively antibiotic-resistant compared to cells grown in suspension. Herein is reported the attachment, growth and antibiotic susceptibility of *M. avium* cells grown in suspension and in catheter biofilms.

METHODS

*M. avium* strain and growth. *M. avium* strain A5, which was originally recovered from an AIDS patient (Beggs et al., 1995), was cultured on Middlebrook 7H10 agar (BBL Microbiology Systems) supplemented with 0.5% (v/v) glycerol and 10% (v/v) oleic acid albumin (M7H10) and incubated for 7 days at 37°C. A single transparent colony was picked and used to inoculate 2 ml Middlebrook 7H9 broth, with the same supplements, in a 16 x 125 mm screw-capped tube. The resulting inoculum culture was incubated at 37°C for 7 days without shaking and streaked on Plate Count Agar (Difco) for contaminants and M7H10 agar to confirm colony morphology. One millilitre of culture was used to inoculate 9 ml M7H9 in a 20 x 150 mm screw-capped tube and incubated at 37°C for 7 days with aeration by rotation to produce exponential phase growth. If the 10 ml culture was found to be free from contaminants and had the correct transparent colonial morphology, it was refrigerated and could be used for up to 2 weeks.

Catheter biofilm apparatus. A CADD-Legacy 1 Ambulatory Infusion Pump model 6400 (SIMS Deltec) with 50 ml reservoir was used for the experiments. The tubing was composed of polyvinyl chloride plasticized with trioctyl trimellitate, acrylonitrile-butadiene-styrene, polycarbonate and silicone, and had an interior diameter of 0.1 cm. The calculated volume and surface area for the tubing was...
0.01 ml and 0.314 cm² cm⁻¹ length. The pump and tubing system was chosen because of its widespread use in the United States. The tubing material is used in catheters from a variety of suppliers.

**Catheter apparatus inoculation.** Following assembly of the infu-

**Antibiotic susceptibility measurements of cells in suspension.** Following 24 h adherence, the reservoir was replaced with sterile tap water after 24 h, and the tubing rinsed before the pump was restarted. Samples were collected from the distal portion of the tubing through which the water or suspension had flowed for the specific time period. In our experience, colonization of mycobacteria on the surface of catheter tubing was not uniform requiring that long sections of catheter tubing (10 cm) were sampled at least twice. That practice resulted in good reproducibility of the measurements from independent experiments.

**Adherence of *M. avium* to catheter tubing**

In Table 1 the mean c.f.u. cm⁻² (±SD) of *M. avium* cells adhering to the catheter tubing at different times for four independent experiments is shown. The only colony types recovered from the adherence measurements were transparent, as was the isolate used for inoculating the
cultures and suspensions. The measurements were limited to 24 h to reduce the impact of any increase in cell number in the reservoir suspension or tubing (suspension and adherent cells) due to growth. After 4 and 6 h of adherence, numbers of *M. avium* equalled those in drinking water biofilms (Falkinham *et al.*, 2001; Torvinen *et al.*, 2004) and artificial systems with other mycobacteria and surfaces (Bardouniotis *et al.*, 2001, 2003; Hall-Stoodley & Lappin-Scott, 1998; Hall-Stoodley *et al.*, 1999; Schulze-Röbbe & Fischeder, 1989) including *M. avium* (Carter *et al.*, 2003). The observation that there was no increase in adherent cells from 4 to 6 h (Table 1) was repeatable and may be due to the possibility that not all cells in the suspension were capable of adherence due to growth stage differences. The number of *M. avium* cells at 24 h could be due to both adherence and growth of adherent cells. Unfortunately, no surface counts were provided in previous reports of catheter-related mycobacterial infections for comparison. The influence of different flow rates or cation and anion concentrations was not investigated.

**Growth of *M. avium*** in catheter biofilms

To measure growth certain precautions were required. The pump was turned off at 24 h and the reservoir switched to one containing only sterile Blacksburg tap water. Furthermore, the entire length of the catheter tubing was rinsed with autoclaved Blacksburg tap water. Thus, increase in numbers due to further adherence and growth of the mycobacterial cells in the reservoir and tubing did not contribute to numbers of adherent cells, and only increase in the number of already adherent cells was measured. Cells of *M. avium* strain A5 grew on the surfaces of the catheter tubing (Fig. 1). The numbers were reproducible and reached very high densities by 4 weeks (almost $7 \times 10^6$ c.f.u. cm$^{-2}$). The uneven distribution of cells and colonies in the catheter tubing undoubtedly contributed to the variation in the c.f.u. values. It is likely that the drop from 24 h (end of adsorption period, Table 1) to 48 h (0 time, Fig. 1) reflected the absence of any contribution of the inoculated reservoir ($2 \times 10^6$ c.f.u. ml$^{-1}$ at 24 h) and rinsing the complete length of the catheter tubing with autoclaved Blacksburg tap water. Only transparent colony types were recovered from the growth measurements, so there was no change or selection for other types (e.g. opaque or rough). The plot of c.f.u. cm$^{-2}$ against duration of incubation (Fig. 1) demonstrated that the increase in *M. avium* strain A5 numbers was exponential over the first 2 weeks of incubation (doubling time 2.45 days) and stationary phase had not been reached by 4 weeks (Fig. 1). This doubling time is remarkable considering that cells were in a medium consisting essentially of autoclaved tap water at 25 °C. The doubling times for cells growing in biofilms have been reported for *M. phlei* (0.4 days; Bardouniotis *et al.*, 2001), *Mycobacterium marinum* (0.45 days; Bardouniotis *et al.*, 2003) and *M. fortuitum* (0.3 and 0.18 days; Bardouniotis *et al.*, 2003 and Hall-Stoodley & Lappin-Scott, 1998, respectively). Although these reported doubling times are considerably shorter, they were measured under conditions where growth of adherent cells and continued adherence of cells from suspension both contributed to the increased accumulation of cells on the surfaces. Further, in all those studies, cells were grown in Middlebrook 7H9 broth at 37 °C, which would substantially increase growth rates.

**Antibiotic susceptibility of *M. avium*** in catheter biofilms

Antibiotic susceptibility was measured against three types of *M. avium* strain A5 cells: (1) cells were grown for 1 week in catheter biofilms and exposed to the antibiotics while in the biofilm (sessile), (2) cells grown for 1 week in biofilms were harvested from catheter tubing (liberated cells) and exposed to the antibiotics in suspension, (3) cells grown in suspension for 1 week were collected from the water flowing through catheter tubing (planktonic cells) and exposed to

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**Table 1. Adherence of *M. avium* strain A5 cells to catheter tubing**

The numbers of *M. avium* cells adhering to catheter tubing upon exposure of tubing to $2 \times 10^6$ c.f.u. *M. avium* ml$^{-1}$ are given. Numbers are expressed as the means (± SD) of four independent experiments.

<table>
<thead>
<tr>
<th>Duration of adherence (h)</th>
<th>No. of adhering cells (c.f.u. cm$^{-2}$ catheter tubing ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$&lt;3.2$</td>
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<tr>
<td>2</td>
<td>9.0 ± 4.4</td>
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<tr>
<td>4</td>
<td>25.6 ± 3.7</td>
</tr>
<tr>
<td>6</td>
<td>30.1 ± 2.1</td>
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<td>24</td>
<td>356 ± 63.3</td>
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**Fig. 1. Growth of *M. avium* strain A5 in catheter biofilms.**

The number of *M. avium* cells is expressed as c.f.u. cm$^{-2}$ following a 24 h adherence period and 24 h circulation of sterile tap water (time 0 = 48 h). Incubation was at 25 °C. Values are expressed as the means (±SD) of two independent experiments.
antibiotics. All cell types were exposed to 100 μg clarithromycin ml⁻¹ or 100 μg rifamycin ml⁻¹ for 0, 1, 2 and 3 h. The results, listed in Table 2 (mean ± SD for two independent experiments), document the variation in numbers of surviving cells exposed to antibiotics in biofilms (sessile) or harvested and suspended (liberated) compared to those of cells grown in suspension (planktonic). The variation in counts (Table 2) is likely due to the presence of aggregates in planktonic and liberated cells that might be incompletely dispersed. For cells exposed to antibiotics in biofilms, the major reason for the variation in numbers is the uneven distribution of cells and microcolonies along the length of the catheter tubing. In spite of this variation, the trends are clear and demonstrate that survival of cells of *M. avium* strain A5 cells grown in biofilms was significantly higher than that of cells in suspension (Student’s *t*-test, *P* < 0.05). There was no significant difference between the susceptibility of cells grown in biofilms and exposed in either the biofilm or in suspension (Table 2). Possibly the relatively low density and immaturity (e.g. absence of an extracellular matrix) of the biofilm was responsible. It is unlikely that aggregation contributed to antibiotic resistance due to reduced penetration of the antibiotics. Microscopic examination of the suspensions showed that the largest aggregates were 25 colonies or fewer and the biofilms consisted of monolayers of cells and small microcolonies (fewer than 100 cells colony⁻¹). Such aggregates would not prevent antibiotic penetration in the time-frames employed here (Nichols et al., 1989). Only transparent colonies were detected amongst the survivors, indicating that antibiotic exposure did not select for an alternative type. This is expected, because the transparent types are more antibiotic resistant than opaque colony types (Woodley & David, 1976). Quite possibly, antibiotic exposure of biofilms of opaque *M. avium* variants might lead to selection for transparent variants. The fact that the cells grown in catheter biofilms and exposed to antibiotics in suspension were as resistant to the antibiotics as were cells exposed in biofilms, suggests that physiological changes, leading to antibiotic resistance, occur in *M. avium* cells when grown on surfaces. The same was noted for cells of *M. avium* grown on glass biofilms and exposed to chlorine (Steed & Falkinham, 2006).

The data demonstrate that cells of *M. avium* adhere to and grow on a type of catheter surface and are consistent with reports of catheter-related mycobacterial infections, including those due to *M. avium* (Schelonka et al., 1994). Although the growth rate of *M. avium* strain A5 was rather slow (2.45 days doubling⁻¹), it was sufficient to generate a substantial biofilm population in tap water after 2 weeks (Table 2). Based on earlier data showing that the majority of *M. avium* cells in drinking water systems are in biofilms on pipe surfaces (Falkinham et al., 2001; Torvinen et al., 2004), our strategy for isolating, detecting and enumerating mycobacteria now focuses on surfaces as the samples of choice.

The fact that cells of *M. avium* grown on catheter surfaces are significantly more resistant to antibiotics than cells grown in suspension is in agreement with similar studies with other pathogens, including *Pseudomonas aeruginosa* (Nickel et al., 1985). Furthermore, the antibiotic-resistance of catheter-biofilm-grown *M. avium* has implications for treatment of infected patients and methods for measuring antibiotic susceptibility of *M. avium* and probably other mycobacteria. Cells of *M. avium* strain A5 grown in biofilms, liberated and exposed to antibiotics in suspension were more resistant than cells grown in suspension (Table 2). In

<table>
<thead>
<tr>
<th>Cell growth and exposure</th>
<th>Duration of exposure (h)</th>
<th>Percentage survival ± SD</th>
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<tr>
<td></td>
<td></td>
<td>Clarithromycin</td>
</tr>
<tr>
<td>Grown and exposed in suspension</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
<td>1</td>
<td>19 ± 3</td>
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<td></td>
<td>2</td>
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<td></td>
<td>3</td>
<td>2 ± 0.7</td>
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<tr>
<td>Grown in biofilms exposed in suspension</td>
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<td>Grown and exposed in biofilms</td>
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<td>79 ± 43</td>
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<td>73 ± 51</td>
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contrast, cells of *P. aeruginosa* grown in biofilms liberated and exposed to antibiotics in suspension were as susceptible to antibiotics as were planktonic cells (Anwar et al., 1989). Thus, it would appear that biofilm growth leads to a physiological change, reflected here as antibiotic resistance. Because cells liberated from catheter biofilms were as resistant to antibiotics as were cells exposed in biofilms, antibiotic concentrations based on results of the susceptibility of suspension-grown cells may be too low to kill or inhibit the growth of mycobacteria released from the catheter biofilms. Consequently, it may be necessary to develop methods for assessing MICs of mycobacteria grown in biofilms.

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**REFERENCES**


