Humic and fulvic acids stimulate the growth of 
**Mycobacterium avium**

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**Abstract**

*Mycobacterium avium*, an environmental, opportunistic pathogenic mycobacterium, has been isolated frequently and in high numbers from waters in Finland and from acid, brown water swamps of the southeastern coastal USA. *M. avium* has also been recovered in high numbers from Finnish drinking water and frequently isolated from Finnish AIDS patients. Boreal forests and brown water swamps are similar in that they are rich in humic and fulvic acids and of low pH and dissolved oxygen. Growth of representative isolates of *M. avium* in natural water was stimulated markedly by the addition of humic and fulvic acids. Further, the *M. avium* isolates grew at pH levels as low at 4.0 and at oxygen levels equal to 4% of atmospheric levels. The high numbers of *M. avium* in boreal waters and brown water swamps are likely due to their ability to proliferate in those humic- and fulvic-rich, acidic, micro-aerobic environments. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Environmental mycobacterium; Humic; Fulvic acid; *Mycobacterium avium*

**1. Introduction**

*Mycobacterium avium* is an opportunistic human and animal pathogen whose source for human infection is the environment [1–3]. Approximately 25–50% of late stage AIDS patients are infected with *M. avium* [4]. The identity of DNA fingerprints of *M. avium* isolates from AIDS patients with those *M. avium* isolates recovered from natural or potable waters to which the patients were exposed [2] is strong proof that water is one source of *M. avium* infection in humans. Mycobacteria, including *M. avium*, have been isolated from a wide variety of natural and potable waters [5–7]. In particular, boreal coniferous forest soils are rich in mycobacteria [8]. Further, boreal coniferous waters [9] and brook [10] and drinking waters [7] collected in Finland and soil and water samples collected from coastal, acidic, brown water swamps of the USA [11] have yielded the highest numbers of mycobacteria.

*M. avium* numbers in the coastal, acidic, brown water swamps correlated with high humic and fulvic acid concentrations, low pH, low dissolved oxygen and high zinc concentrations [11]. The higher numbers of mycobacteria in samples rich in humic and
fulvic acids agree with the demonstration that the occurrence of mycobacteria in Finland correlated positively with the presence of peatlands and a low pH [9].

Herein, we test the hypothesis that the abundance of M. avium in drinking water in Finland and in Finnish peatlands and in the coastal brown water swamps of the USA is due to the stimulation of the growth of these environmental opportunistic pathogens by humic and fulvic acids. A second aim was to demonstrate that M. avium isolates can grow at the low pH and low oxygen levels characteristic for those environments.

2. Materials and methods

2.1. Mycobacterial strains

Table 1 lists the M. avium strains used in this study and their source of isolation.

2.2. Growth of mycobacterial strains

M. avium strains were grown in filter-sterilized (0.2-μm pore size) natural water samples collected from Claytor Lake (an Appalachian fresh-water lake in Virginia, humic acid = < 0.10 mg l⁻¹, fulvic acid = < 0.5 mg l⁻¹, pH = 7.2–7.6) or Lake Drummond (an acidic, brown water swamp in the Great Dismal Swamp in Virginia, humic acid = 41 mg l⁻¹, fulvic acid = 345 mg l⁻¹, pH 4.5–5.0) to the mid-exponential phase (1 × 10⁷ colony-forming units (CFU) ml⁻¹) at 37°C with aeration. Though the strains formed aggregates during growth, the aggregates could be dispersed by 60 s vortexing, permitting accurate measurement of absorbance.

2.3. M. avium growth response studies

Solubilized, filter-sterilized humic or fulvic acids, isolated from Dismal Swamp soils isolated as described in [12] or purchased (Aldrich Chemical, Milwaukee, WI, USA), were added to filter-sterilized Claytor Lake water to achieve concentrations equal to those of Lake Drummond. Controls included cultures grown in filter-sterilized Claytor Lake water. Cultures of the strains grown in Claytor Lake water samples were used as inocula to reduce the transfer of organic material. Following inoculation, there were between 10⁷–10⁸ CFU ml⁻¹. To measure the effect of a reduced oxygen level, a vacuum desiccator was evacuated to 0.2 atmospheres partial pressure and nitrogen gas was used to replace the air. The influence of pH on the growth of the M. avium strains was examined by adjusting the pH of unsupplemented Claytor Lake water to 4.0, 5.5, 7.0 and 8.5 with either diluted HCl or NaOH. To examine the interaction of humic and fulvic acid addition and the oxygen level, strains were grown in sterile Claytor Lake water incubated at a normal oxygen tension, sterile Claytor Lake water containing 0.05 mg humic acid ml⁻¹ and 0.4 mg fulvic acid ml⁻¹, incubated at the reduced oxygen tension (i.e. approximately 4%), and sterile Lake Drummond water under reduced oxygen tension. Growth was measured by the increase in absorbance at 580 nm, a wavelength minimizing the absorption of light by the yellow pigment.

Table 1

<table>
<thead>
<tr>
<th>M. avium strain</th>
<th>Strain origin</th>
<th>Nil</th>
<th>Growtha</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Humic acid</td>
<td>Fulvic acid</td>
<td></td>
</tr>
<tr>
<td>2812P</td>
<td>AIDS patient</td>
<td>0.066</td>
<td>0.151*</td>
</tr>
<tr>
<td>2279</td>
<td>Chicken</td>
<td>0.008</td>
<td>0.020*</td>
</tr>
<tr>
<td>13S</td>
<td>Aerosol droplet</td>
<td>0.123</td>
<td>0.164*</td>
</tr>
<tr>
<td>DSW69</td>
<td>Water, Dismal Swamp</td>
<td>0.193</td>
<td>0.272*</td>
</tr>
<tr>
<td>OKS41</td>
<td>Soil, Okefenokee Swamp</td>
<td>0.117</td>
<td>0.240*</td>
</tr>
<tr>
<td>OKW75</td>
<td>Water, Okefenokee Swamp</td>
<td>0.129</td>
<td>0.217*</td>
</tr>
</tbody>
</table>

*Growth expressed as the increase in absorbance at 580 nm after 10 days incubation of 5 ml culture in 16×125-mm screw-capped tubes. Humic and fulvic acids were isolated from Dismal Swamp soils. The values represent averages of three independent experiments.

*Values significantly different (P < 0.05) from control by Duncan’s multiple range test.
of some strains. Because of the absorbance of 580-nm light by humic and fulvic acids, an uninoculated blank was used and growth was reported as the increase in absorbance. Following incubation, cultures were streaked to ensure purity. In all experiments reported here, pure cultures of the inoculated strains with the same colonial morphology were obtained.

2.4. Statistical analysis

Duncan’s multiple range test or Student’s t test of the Statistical Analysis Systems program [13] were performed to compare differences in growth of isolates. For significance, a confidence interval of 95% was selected for both statistical procedures.

3. Results

3.1. Stimulation of M. avium growth by humic and fulvic acids

Growth of all six M. avium strains in Claytor Lake water was significantly increased ($P < 0.05$) by either 0.05 mg humic acid $l^{-1}$ or 0.4 mg fulvic acid $l^{-1}$ (with one exception, strain 13S) at 37°C (Table 1) or 16°C (data not shown). The humic and fulvic acids were isolated from Dismal Swamp soils. All the M. avium strains grew to different extents in the unsupplemented Claytor Lake water (Table 1 and Fig. 1), in confirmation to published data [14]. The data in Table 1 reflect growth stimulation of cultures in tubes, whereas the data in Fig. 1 reflect growth in flasks where there was more aeration (S.D.s of the average growth increments were less than 0.020). Humic and fulvic acids isolated from the Okefenokee Swamp (GA, USA), Dismal Swamp and a commercial supplier (Aldrich Chemical) did not differ to the extent of M. avium growth stimulation (data not shown).

The growth response of M. avium isolates to different concentrations of humic or fulvic acids was also measured. The increase in turbidity of cultures of the four M. avium strains tested was significantly greater in the presence of either humic or fulvic acids and greater at the higher concentrations of either humic or fulvic acid for three of the four isolates tested (Table 2). The data in Table 2 demonstrate that 0.4 mg fulvic acid $l^{-1}$ can stimulate growth of M. avium strain 13S in flask cultures where higher oxygen levels are present compared to tube cultures (i.e. Table 1).

3.2. Effect of pH on growth

The M. avium strains grew over a wide pH range

Table 2

<table>
<thead>
<tr>
<th>Compound concentration$^a$</th>
<th>Growth$^b$ of M. avium strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OKS41</td>
</tr>
<tr>
<td>Nil</td>
<td>0.030</td>
</tr>
<tr>
<td>Humic acid</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>0.078*</td>
</tr>
<tr>
<td>0.05</td>
<td>0.108*</td>
</tr>
<tr>
<td>0.5</td>
<td>0.130*</td>
</tr>
<tr>
<td>Fulvic acid</td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>0.085*</td>
</tr>
<tr>
<td>0.4</td>
<td>0.200*</td>
</tr>
<tr>
<td>4.0</td>
<td>0.150*</td>
</tr>
</tbody>
</table>

$^a$Concentrations listed as mg humic or fulvic acid $l^{-1}$ of culture.
$^b$Growth expressed as the increase in absorbance (580 nm) after 10 days incubation. The values represent averages of three independent experiments.
$^*Values significantly different ($P < 0.05$) from control by Duncan’s multiple range test.
in Claytor Lake water samples (Table 3). The weakly turbid cultures contained approximately $10^7$ CFU ml$^{-1}$. The growth kinetics of $M. avium$ strain 13S in unsupplemented Claytor Lake water samples adjusted to pH 4.0, 5.5, 7.0 or 8.5 are shown in Fig. 2 (S.D.s of the average growth increments were less than 0.020). Both the growth rate and final turbidity were lower in Claytor Lake water adjusted to pH 8.5, compared to the other pHs. Though the data in Fig. 2 came from a water sample incubated at 37°C, similar results (albeit at a slower growth rate) were obtained for a culture incubated at 16°C (results not shown). The water temperature of the acidic, brown water swamps in the southeastern USA is 45°C during the summer months (Parker and Falkinham, unpublished observation).

3.3. Effect of reduced oxygen tension on $M. avium$ growth in the presence of humic and fulvic acids

We next sought to determine whether the growth stimulation of $M. avium$ isolates by humic and fulvic acids occurred under the reduced oxygen levels characteristic of peatlands and acidic, brown water swamps. Growth of $M. avium$ isolate 13S in Claytor Lake water was significantly higher ($P < 0.05$) in Claytor Lake water to which humic and fulvic acids were added and in Lake Drummond water, both incubated at the reduced oxygen level (Fig. 3, S.D.s of the average growth increments were less than 0.02). There was no difference between the growth of strain 13S in Lake Drummond water incubated at reduced (Fig. 3) or normal oxygen tensions (data not shown). Possibly, compounds other than humic and fulvic acids are resulting in oxygen-independent growth stimulation (e.g. nitrate). The other five $M. avium$ isolates showed a similar response (results not shown).

![Fig. 2. Growth (change in absorbance at 580 nm) of $M. avium$ isolate 13S in sterile Claytor Lake water adjusted to pH 8.5 (•), 7.0 (○), 5.5 (△) and 4.0 (□) at 37°C. Values are means of triplicate cultures.](image)

![Fig. 3. Growth (change in absorbance at 580 nm) at 37°C of $M. avium$ isolate 13S in 10 ml sterile Claytor Lake water incubated under normal oxygen tension (•), 10 ml sterile Claytor Lake water containing 0.05 mg humic acid ml$^{-1}$ and 0.4 mg fulvic acid ml$^{-1}$ incubated under reduced oxygen tension (△) and 10 ml sterile Lake Drummond water incubated under reduced oxygen tension (○) in 125-ml flasks. Values are means of triplicate cultures.](image)

### Table 3

<table>
<thead>
<tr>
<th>$M. avium$ isolate</th>
<th>Growth* in Claytor Lake water at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td>2812P</td>
<td>0.061</td>
</tr>
<tr>
<td>2279</td>
<td>0.004</td>
</tr>
<tr>
<td>13S</td>
<td>0.086</td>
</tr>
<tr>
<td>DSW69</td>
<td>0.219</td>
</tr>
<tr>
<td>OKS41</td>
<td>0.143</td>
</tr>
<tr>
<td>OKW75</td>
<td>0.133</td>
</tr>
</tbody>
</table>

*Growth expressed as the increase in absorbance at 580 nm after 10 days incubation. The values represent averages of three independent experiments. S.D.s were between 0.005 and 0.013.
4. Discussion

Growth of representative clinical and environmental M. avium strains in natural waters was stimulated by humic and fulvic acids alone (Fig. 1 and Tables 1 and 2) or in combination (Fig. 2). The concentrations chosen were similar to those measured in the waters of Lake Drummond, an acidic, brown water lake in the Dismal Swamp of Virginia. Growth of the M. avium isolates was greater at higher concentrations of both humic and fulvic acids (Table 2). This is the first report demonstrating that growth of M. avium strains is stimulated by humic and fulvic acids alone or in combination. The differences in response to addition of either humic or fulvic acids likely reflects the diversity of M. avium strains [3].

We do not yet have an explanation for the growth stimulation of M. avium by humic and fulvic acids. Humic and fulvic acids are of a high to low molecular mass and contain a wide variety of aromatic and aliphatic structures bearing many carboxy and alcoholic groups, permitting them to behave as polyelectrolytes [15–18]. Fulvic acids are acid-soluble, whereas humic acids are acid-insoluble [15–18]. They do not appear to stimulate growth by providing a substrate for energy, because oxygen consumption by the five strains was not stimulated above endogenous rates by the addition of either humic or fulvic acids (data not shown). Humic acids can serve as electron acceptors for microbial respiration [15] and anaerobic microbial oxidation [16]. Possibly, mycobacteria can oxidize and degrade humic and fulvic acids for carbon and nitrogen while transferring electrons to humic and fulvic acids without stimulating oxygen consumption. Possibly also, the growth stimulation is due to the chelation of metals by humic and fulvic acids [17]. The number of M. avium in the acid, brown water swamps correlated with the concentration of zinc [10]. The ability of humic acids to alter partition coefficients [18] may also contribute to the growth stimulation. Detergents, that also influence partition coefficients, increase the permeability and growth of mycobacteria, including M. avium [19].

M. avium complex strains also grew in natural waters over a wide pH range (Fig. 2 and Table 2). This work confirms the earlier demonstration that M. avium strains could grow in unsupplemented natural waters [14]. Previous reports had also shown that M. avium strains could grow over a wide pH range in laboratory media, though growth was reduced at alkaline pHs [20,21]. Those reports and the data presented here emphasize that M. avium strains from diverse origins grew as well at acidic pH values as at neutral pH (Fig. 2 and Table 3).

Growth stimulation of the M. avium isolates by humic and fulvic acids occurred in natural waters whether the cultures were incubated under normal or reduced oxygen tensions (Fig. 3). Thus, humic or fulvic acid growth stimulation did not require a highly aerobic environment.

Previous investigations established that mycobacterial numbers were high in natural and drinking water, peatlands and boreal coniferous forest soils of Finland [7–10] and M. avium numbers were high in acid, brown water swamps of the coastal USA [11]. Further, mycobacterial numbers correlated with the presence of peatlands and water color (an indicator of the presence of the brown humic and fulvic acids) [10]. M. avium numbers correlated with a low pH, reduced oxygen tension and the presence of humic and fulvic acids [11]. Thus, we conclude that the ability of M. avium to grow at a low pH and low oxygen tension, coupled with the growth stimulation provided by humic and fulvic acids, are physiologic factors influencing the ecology, geographic distribution and epidemiology of these environmental, opportunistic human and animal pathogens. Clearly, M. avium is not a contaminant, but is a natural component of such ecosystems. It is likely that regions where drinking water sources lie in either boreal coniferous forests or in acid, brown water swamps, drinking water may contain substantial numbers of M. avium, as is the case in Finland [7].

Acknowledgements

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References


