Studies of Microthrix parvicella in situ and in laboratory culture: production and use of specific antibodies

N. Connery*, A.S. Thompson†**, S. Patrick** and M.J. Larkin*

* The QUESTOR Centre & School of Biology and Biochemistry, The Queen’s University of Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, United Kingdom
** Department of Microbiology and Immunobiology, School of Medicine, The Queen’s University of Belfast, Belfast. BT12 6BN, United Kingdom
† Communicating author (E-mail: a.s.thompson@qub.ac.uk)

Abstract Physiological studies on M. parvicella have been conducted to determine the rate of growth of this organism in pure culture. The organism displayed a doubling time of 128 days despite its profuse abundance in a local Wastewater Treatment Plant (WWTW). An extensive survey has been ongoing since February 2000 into the extent of M. parvicella in the WWTW. A suite of monoclonal and polyclonal antibodies has been developed to detect and quantify M. parvicella.

Keywords Activated sludge; bulking; immunofluorescence; Microthrix parvicella

Introduction

In the last ten years M. parvicella has been recorded as being the most frequently present filamentous micro-organism in bulking sludge in Europe (Eikelboom et al., 1998; Wanner et al., 1998). Its presence in biological nutrient removal (bio-NR) plants is seasonal and appears to be stimulated by temperature and dissolved oxygen (DO) concentration. Despite its profuse abundance in bulking sludge during winter and spring, it is slow growing in pure culture and has an optimum growth temperature of 12°C–15°C (Knoop and Kunst, 1998) making biochemical and physiological studies difficult. In addition, its filamentous nature means that it is difficult to enumerate by conventional direct means.

Immunological methods have been reported for the detection of specific populations of filamentous and non-filamentous bacteria in wastewater systems other than M. parvicella (Howgrave-Graham and Steyn, 1988 and Raskin et al., 1998). M. parvicella has been detected in sludge using fluorescent in situ hybridisation (FISH) (Erhart et al., 1997) but with variable results, and requiring a methodology not suitable for routine analysis within the water industry. The relative ease with which the signal produced using immuno-technologies offers the possibility of quantitative immunofluorescence microscopy (IFM) to complement diagnostic FISH, and provide the basis of quick quantitative and qualitative analyses.

We describe here the results of a long-term survey of municipal WWTWs within Northern Ireland, a number of physiological growth experiments on pure cultures of M. parvicella and the production and use of a range of antibodies specific to M. parvicella.

Methods

Microthrix parvicella RN1 (Rossetti et al., 1997) was obtained from Dr. Valter Tandoi (Water Research Institute, Rome). The growth medium (R2AM) used was a modified form of R2A (Reasoner and Geldreich, 1985), with the additions of (mg per litre medium): CaCl₂·2H₂O, 50; NaEDTA, 50; Na₂MoO₄, 20; cycloheximide; 20, NMS Trace Elements (Atlas, 1995), vitamins (Eikelboom, 1968). Supplements to this medium, where mentioned, included centrifuged sludge, filter sterilised (0.2 μm) or autoclaved, filter
sterilised spent *M. parvicella* culture supernatant. Cell quantification was by microBCA Protein Assay Kit (Pierce), spectrophotometric analysis (optical density of cultures @550 nm, in microtitre plates).

Polyclonal antibodies and monoclonal antibodies were produced from whole live cells according to previously published methods (Lutton *et al.*, 1991). IFM followed the methods previously reported (Ramage *et al.*, 1998), using methanol fixed cells.

**Results and discussion**

**Survey of WWTWs**
A 12-month survey regarding the extent of *M. parvicella* in a municipal WWTW, was carried out from February 2000. This involved the measurement of a number of physical and chemical parameters on a weekly basis (Figure 1). It was found that as temperature increased seasonally, the volume of suspended solids (VSS, in mls) did not reduce, as found elsewhere (Knoop and Kunst (1998), Mamais *et al.* (1998)) with the exception of several samples from October 2000 which were investigated (Connery *et al.*, in preparation). Indeed, this plant has exhibited poor settling continuously during this study, which began in September 1999. Quantities of *M. parvicella* filaments in these samples are seen to follow the trend observed for settling (data not published).

**Physiological studies**

Despite the abundance of *M. parvicella* in the WWTW sludge, RN1 has been found to grow slowly in pure culture, with a doubling time of 128 days (Figure 2). It has been shown that the culturability of aged *Mycobacterium tuberculosis* cultures is greatly improved when grown in spent culture supernatant (Sun and Zhang, 1999) and the effect of “autoinducing” compounds on Actinomycetes has now been widely reported (see Horinouchi and Beppu, 1992). Some of the work reported here investigated the existence of such a factor (be it an autoregulatory compound or one which is indigenous to sludge), to explain the slow growth observed in pure culture. Typical growth yields can be seen in Table 1 for *M. parvicella*.

The organism was grown on R2AM medium and in R2AM supplemented with sterilised sludge supernatant obtained from the WWTW on 8/3/2000. This sludge sample did not improve the growth yield beyond that of the non-supplemented medium, R2AM. Figure 3

**Table 1** Growth yields expressed as total protein of *M. parvicella* in sludge-supplemented media. (*n* = 5, *p*<0.05)

<table>
<thead>
<tr>
<th>Medium A</th>
<th>Medium B</th>
<th>Medium C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sludge supernatant</td>
<td>Sludge supernatant</td>
<td>–</td>
</tr>
<tr>
<td>Glucose, Pyruvate</td>
<td>–</td>
<td>Glucose, Pyruvate</td>
</tr>
<tr>
<td>R2AM base</td>
<td>–</td>
<td>R2AM base</td>
</tr>
<tr>
<td>166.0 µg/ml ± 4.09</td>
<td>169.3 µg/ml ± 4.09</td>
<td>175.1 µg/ml ± 6.03</td>
</tr>
</tbody>
</table>

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**Figure 1** Volume of Suspended Solids (VSS) vs. temperature of sludge

**Figure 2** Regression of OD (550 nm) of *M. parvicella* in microtitre plate wells (R2A, *n* = 16)
illustrates a similar experiment using a sludge supplement obtained on 2/2/2000 (bars 3 and 4) which indicates that some of these supplements may actually inhibit growth. Growth of *M. parvicella* in R2AM medium supplemented with the supernatant of spent culture was also examined (Figure 3). When grown in the presence of re-used culture medium, growth yields were not significantly increased.

**Detection of filaments in sludge by IFM**

Polyclonal and monoclonal antibodies have been obtained and characterised (Thompson et al., in preparation). A range of mixed slurries from activated waste treatment systems were examined for *M. parvicella* using conventional means (Eikelboom, 1968) and IFM. All the antibodies described detected natural populations of *M. parvicella* in samples, though some showed brighter fluorescence across a range of samples than others. A cocktail of the monoclonal antibodies was found to be the most reliable method of detecting filaments *in situ*, as the intensity of the autofluorescing background material often reduced the clarity of spe-
specific antibody staining when only one monoclonal was used. On the basis of IFM analysis, *M. parvicella* was found to be the dominant filament within the WWTW and indeed, it was the dominant microorganism. We also observed different patterns of fluorescence within the same works WWTW at different sampling locations (Figure 4).

Samples were drawn from different locations within the same aerated Carrousel-type system; from a non-aerated (“anoxic”) section, mid-aerated and from the final point before discharge to settling tanks (the point where maximum aeration might be expected). The polyclonal antibody showed increasing fluorescence with an increase in aeration. It is possible that this was the result of increased expression of an epitope associated with oxidative metabolism. It is also notable that it was possible to elucidate a fine structure within *M. parvicella* in sludge samples, particularly in brightly fluorescing examples.

Conclusions

*M. parvicella* has been seen to be present all year round in this WWTW as determined from February 2000 to February 2001. This plant has observed the phenomenon of bulking since February 2000. The growth yield of *M. parvicella* is not improved when grown in the presence of sludge from this plant obtained on either February 2nd or March 8th, 2000. The same may be said for the addition of spent *M. parvicella* culture supernatant. The organism is slow growing with a doubling time of 128 days. A range of specific antibodies has been produced against *M. parvicella* RN1 which have been shown to show varying reactivity against putative *M. parvicella* filaments found in the WWTW.

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References


