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BACTERIA ASSOCIATED WITH WELL WATER: BIOGEOCHEMICAL TRANSFORMATION OF Fe AND Mn, AND CHARACTERIZATION AND CHEMOTAXIS OF A METHYLOTROPHIC HYPOMICROBIUM SP.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Laura Tuhela, B.S., M.S.

* * * * *

The Ohio State University

1995

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ACKNOWLEDGMENTS

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FIELD OF STUDY

Major Field: Microbiology
Studies in: Microbial Ecology
Fe Biogeochemistry
Biodegradation
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SCOPE OF THE WORK

There are primarily two major sections to the scope of the work presented in the following pages. The sections are related because they deal with bacteria present in or isolated from water wells with previous histories of Fe/Mn biofouling.

Chapter I is a review of biogeochemical transformations of Fe and Mn which occur in water wells and the bacteria that have been previously implicated in these transformations. The work presented in Chapters II and III is concerned with Fe-precipitating bacteria, biofilms with which these bacteria are associated, and Fe-precipitates formed in the presence of these bacteria.

The second section of this work involves the appendaged bacteria that were present in many of the biofilm samples retrieved from the water wells. Chapter IV reviews several genera of appendaged bacteria. The enrichment and characterization of the appendaged bacteria from the water well samples are described in Chapter V. One of the isolates obtained in this study was further characterized with respect to the motility, chemotaxis, and structure of the flagella (Chapter VI).

The work in Chapter VI has led to interesting questions involving the flagellin proteins of Hyphomicrobium W1-1B. Some of these future research topics are presented in the Concluding Remarks following Chapter VI.
CHAPTER I

TRANSFORMATIONS OF FE AND MN IN WATER WELLS

Abstract

Dissolved Fe(II) and Mn(II) are usually oxidized to poorly soluble mineral species when groundwater is exposed to surface conditions. In water wells, these transformations may eventually result in biofouling problems and deterioration of water quality. Fe(II)-oxidation products include poorly ordered Fe(III)-oxides, such as ferrihydrite, which may be converted to better-ordered Fe(III)-oxides (e.g., hematite) or Fe(III)-oxyhydroxides (e.g., goethite) with time. Among the Mn-oxides, a typical oxidation product is vernadite which is structurally analogous to poorly ordered ferrihydrite. Bacteria implicated in Fe- or Mn-oxidation in neutral pH environments include Gallionella ferruginea, Crenothrix, Sphaerotilus, Leptothrix, and Metallogenium spp. Pedomicrobium, Hyphomicrobium, Clonothrix, Toxothrix, Siderocapsa spp. Magnetotactic bacteria have also been implicated in Fe- and Mn-oxidation although Hyphomicrobium spp. are not capable of Fe- or Mn-oxidation. Very little has been reported on the genera Crenothrix, Clonothrix, Toxothrix, and
Siderocapsa. Research has focused on the stalked and sheathed bacteria Gallionella, Leptothrix, and Sphaerotilus, and a review of available information concerning these genera is presented.

Introduction

Design problems and poor performance are not uncommon in wells used for water supply or for monitoring and contaminant plume control. In recovery and pump-and-treat systems, poor performance is readily observed in terms of reduced flow, increased drawdown, and clogging of downstream piping and treatment apparatus.

Symptoms of performance problems may be less obvious in monitoring wells because they are not continuously stressed by pumping as compared with wells used for water treatment. In both cases, increased drawdown and reduced output may be due to the deposition of mineral precipitates. Changes in water quality may also be involved.

Biofouling and plugging problems are often associated with encrustations and corrosion resulting from the oxidation and precipitation of Fe and Mn. Corrosion tubercles may also have active sulfate-reducing interior zones. An associated problem sometimes attributed to Fe bacteria is the well-filter effect, where pumped water samples may not be representative of the water quality in the aquifer. In-well corrosion of pumps, accompanied by increased turbidity of well water and loss of pump performance, is caused by chemically aggressive waters with elevated concentrations of CO₂, H₂S, or Cl⁻, or extensive biofouling on metallic surfaces. Corrosion problems are
often aggravated by poor material selection for installations.

Biofouling involving the chemical transformation of Fe and Mn is a major factor in well deterioration. The purpose of this paper is to present a summary of geochemical and microbiological transformations of Fe and Mn relevant to water well situations. The extent of Fe and Mn biofouling depends on environmental, hydraulic, and water-use related factors in the well or the downstream receiver. In some cases, symptoms of well deterioration may not be apparent until well performance is severely impaired unless the results of system water quality and performance monitoring are compared over time. Current experience indicates that clogging, biofouling, and Fe and Mn transformations may extend several meters away from existing well holes. New well construction may serve to only temporarily alleviate the problem.

Groundwater treatment plants can be designed to exploit the biologically mediated oxidation of Fe and Mn. Biological removal is usually accomplished in a cascade aeration system followed by infiltration (sand and gravel) to remove the precipitated Fe and Mn (Mouchet, 1992). Large amounts of sheathed, Fe- and Mn-oxidizing bacteria can normally be found in the precipitates. Although biological oxidation is now employed in a number of groundwater treatment plants for Fe and Mn removal, there has been little progress in defining the biology of the microorganisms involved in the oxidation process. A good understanding of the microbial ecology associated environmental effects should facilitate the design of more efficient approaches to Fe and Mn removal and provide solutions to biofouling and corrosion
problems. The purpose of this paper is to summarize the available information on Fe and Mn transformations as they relate to groundwater environments.

**Fe and Mn in Water Wells**

Iron and Mn are released from soils, sediments, and rock by weathering processes. They occur in groundwater as dissolved Fe$^{2+}$ and Mn$^{2+}$ or as various inorganic or organic complexes. Groundwater usually has a molar excess of Fe over Mn. Hatva (1989) reported an average molar ratio of 5.8 for Fe:Mn in a study of 276 water well sites.

Computed Eh-pH diagrams can be used to predict geochemical stability regions for Fe and Mn. Figure 1 presents an Eh-pH diagram for the Fe-O-H system which combines stable solid phases with biologically produced metastable species. Superimposed on Figure 1 are empirical data from water well studies representing several aquifer conditions (Hatva, 1989). At pH near 7, Fe$^{2+}$ is oxidized at an Eh$_{\text{SHE}}$ of 100-200 mV, whereas Mn$^{2+}$ is oxidized at Eh$_{\text{SHE}}$ 600-800 mV (Skinner and Fitzpatrick, 1992). Ferrous iron in groundwater is therefore readily oxidized when it comes in contact with dissolved O$_2$, whereas conditions for the oxidation of Mn$^{2+}$ are seldom achieved abiotically. Sometimes, differential oxidation can lead to a relative enrichment of dissolved Mn$^{2+}$ as Fe$^{2+}$ is oxidized and precipitated in groundwater. The higher the pH the more rapid is the abiotic oxidation of Fe$^{2+}$. Conversely, the lower the pH the more probable it is that the oxidation must be mediated by microorganisms.
Figure 1. Eh-pH diagram for the system Fe-O-H (25°C, 1 atm). Stable solid phases are goethite and magnetite. The dotted line represents a metastable system (*italics*), involving biologically mediated iron oxidation, with ferrihydrite replacing goethite. The diagram is modified from Skinner and Fitzpatrick (1992). The shaded field represents Eh-pH values measured by Hatva (1989) in water well studies of several aquifer conditions. About 85% of Hatva's data (total 276) fall in the shaded field.
In extreme cases, groundwater may be polluted by acid mine seepage, and the resulting low-pH, high-sulfate environment is relatively selective for acidophilic S- and Fe-oxidizers. Williamson et al. (1992) suggested that the combined rate of the bacterial and chemical oxidation of Fe was independent of pH in the range of 2.5 to 6, whereas at pH >6 the rate of oxidation accelerated with increasing pH. The rate of strictly biological oxidation decreases with increasing pH but the rate of chemical oxidation of Fe shows the opposite trend.

Locally high Fe- and Mn-concentrations in groundwater may necessitate treatment to alleviate problems associated with the consumption of Fe- and Mn-rich water. Fe and Mn removal in groundwater treatment plants can be accomplished through aeration which results in the oxidation and precipitation of several solid phases. These precipitates are composed principally of Fe- and Mn-oxides with varying degrees of crystallinity. Morphologically, Fe- and Mn-oxides also vary in size and shape. In groundwater treatment plants, plugging problems in infiltration systems are usually due to colloidal Fe-oxides. Mn-oxides are generally not problematic in this respect because of their tendency to form coarse-grained aggregates. Both types of oxide also have a high sorptive capacity for many metals and oxyanions; thus Fe- and Mn-oxides may become enriched in potentially toxic trace elements. Variations in the elemental composition of Fe- and Mn-precipitates collected from several groundwater treatment plants are shown in Table 1. Several of these samples contained elevated levels of trace elements, probably reflecting variations in geological conditions within the aquifers.
**Fe-oxides**

Ferric iron is soluble only at low pHs (<2.5) typical of acid mine drainage or other acid sulfate waters. At pH 5-7, typical of most groundwaters, Fe\(^{3+}\) is immediately hydrolyzed and precipitated in the form of various oxides and oxyhydroxides (Schwertmann and Fitzpatrick, 1992). In many Eh-pH diagrams, a general or generic stability field for \("\text{Fe(OH)}_3"\) is often used to represent a number of Fe-oxide and -oxyhydroxide minerals. Although most of those minerals are metastable, their formation may be kinetically favored, as shown schematically in Figure 2.

Ferrihydrite (nominally 5Fe\(_2\)O\(_3\)-9H\(_2\)O) is an Fe-oxide commonly found in water wells and groundwater treatment plants (Carlson et al., 1980; Carlson and Schwertmann, 1981; Hatva et al., 1984; Carlson and Schwertmann, 1987). It is poorly crystallized and, therefore, difficult to identify in most natural samples. Ferrihydrite was referred to as amorphous Fe(OH)$_3$ before it was better defined with the advent of improved x-ray diffraction (XRD) techniques. Ferrihydrite usually occurs as small (2-5 nm), rounded and highly aggregated particles that are reddish-brown in color. Structurally better ordered ferrihydrites show six broad XRD peaks (6-line ferrihydrite) whereas the most poorly ordered only yield two diffracted maxima (2-line ferrihydrite).

It has been shown in laboratory experiments (Schwertmann et al., 1984) that the formation of ferrihydrite is favored by the rapid hydrolysis and precipitation of Fe(III). The presence of silicate in synthesis solutions or natural waters tends to stabilize
Table 1. Elemental composition of samples containing Fe- and Mn-precipitates collected from several groundwater treatment plants (A. Vuorinen, University of Helsinki, personal communication).* Background information to these samples has been presented by Hatva et al. (1984) and Carlson and Schwertmann (1987).

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Fe %</th>
<th>Ti %</th>
<th>Mn ppm</th>
<th>B ppm</th>
<th>Co ppm</th>
<th>Cu ppm</th>
<th>Mo ppm</th>
<th>Ni ppm</th>
<th>Sc ppm</th>
<th>Sr ppm</th>
<th>V ppm</th>
<th>Zr ppm</th>
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<td>28.7</td>
<td>6.00</td>
<td>5</td>
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<td>300</td>
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<td>5</td>
<td>555</td>
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*The letter codes refer to different treatment plants. Fe and Mn were analyzed by flame-AAS, the other elements were determined by optical emission spectroscopy. Sorbed and loosely bound impurities were dissolved in 1 M ammonium acetate, pH 4.8, followed by rapid dissolution of Mn-oxides in 1 M hydroxyl ammonium chloride using ultrasonication. Dissolution of Fe(III)-oxides was performed in 1 M hydroxylammonium chloride and 2 M acetic acid. The insoluble residues were composed of silicates. The concentrations have been calculated as cumulative totals from the selective dissolution steps. Fe(III)-precipitates were mainly composed of ferrhydrite and goethite. Vermaditc and birnessite were identified in the Mn(IV)-rich phases.
Figure 2. Pathways of Fe oxide formation and transformation under groundwater aquifer conditions. The scheme is modified from Murray (1979).
ferrihydrite and inhibit the formation of better-ordered Fe-oxide minerals. These observations are in keeping with field observations that ferrihydrite is the most common Fe-oxide mineral in well waters where silicates are invariably present (Carlson and Schwertmann, 1987).

Goethite, α-FeOOH, is probably the most thermodynamically stable form of Fe-oxide under most field conditions. Ferrihydrite tends to transform (via solution) to goethite but the transformation can be extremely slow in the presence of dissolved Si, dissolved organic carbon (DOC), or other inhibitors of crystallization. Goethite is typically formed through slow hydrolysis of Fe\(^{3+}\) released from parent ferrihydrite, and bicarbonate has been shown to favor its crystallization over lepidocrocite (Carlson and Schwertmann, 1990). Minor amounts of goethite are usually found with ferrihydrite in ochreous precipitates from springs and treatment plants (Carlson and Schwertmann, 1987).

Lepidocrocite, γ-FeOOH, is formed upon rapid oxidation and hydrolysis of Fe\(^{2+}\). In a series of experiments with artificial groundwater, lepidocrocite was formed when Fe\(^{2+}\) (added as FeCl\(_2\)) was oxidized through fast aeration (Schwertmann et al., 1984). When silicate ion or DOC was added to the system, Fe precipitated as ferrihydrite instead of lepidocrocite. These results are in agreement with the presence of ferrihydrite in many exposed groundwater environments. Lepidocrocite can usually form only in springs and wells in lateritic soils which are relatively poor in silicates because of their past history of severe weathering.
Other Fe-oxyhydroxides, akaganéite (β-FeOOH) and feroxyhyte (δ'-FeOOH), are rare in nature and not relevant to most exposed groundwater and water well situations. Sulfate complexes of Fe$^{3+}$ (schwertmannite Fe$_6$O$_8$(OH)$_6$SO$_4$; jarosite MFe$_3$(SO$_4$)$_2$(OH)$_6$, where M = K$^+$, Na$^+$, H$_3$O$^+$, or NH$_4^+$) are absent in neutral pH systems. Their stability fields are restricted to acidic waters (pH 3-4 for schwertmannite, pH<3 for jarosites) with high sulfate contents (Bigham et al., 1992, 1994).

**Mn-oxides**

The processes that lead to the formation of different Mn-oxides are not well understood. The stoichiometric composition of Mn-oxides varies, and most Mn-oxides in surface environments also contain some Mn(III) and other cations (Ca, Mg, Na, K, Li, Ba, Al) in addition to Mn(IV).

Birnessite, Na$_{0.7}$Ca$_{0.3}$Mn$_7$O$_{14}$·2.8H$_2$O has been detected most frequently in soils and nodules (Dixon and Skinner, 1992). Birnessite has poor crystallinity and small grain size. Because of its net negative surface charge, birnessite attracts cationic trace metals, as do most Mn-oxide minerals. It is common in bands or lamellae formed in interfacial environments such as those obtained where permeable sediments in the capillary fringe zone are periodically exposed to the groundwater table. Birnessite has also been detected in sand filters in groundwater treatment plants (Hatva et al., 1984; Carlson and Schwertmann, 1987).
Vemadite, $\delta$-MnO$_2$, has short-range order with only two broad XRD-lines and is structurally analogous to 2-line ferrihydrite. Vemadite is difficult to chemically differentiate from other Mn-oxides, especially birnessite. At present, it is doubtful whether vemadite is a distinct minerals species and may be better described as birnessite of extremely small crystal size with a disordered structure (Post, 1992).

Todorokite, $\text{(Ca,Na,K)}_{0.3-0.5}(\text{Mn}^{4+}\text{Mn}^{3+})_6\text{O}_{12} \cdot 3.5\text{H}_2\text{O}$, varies in chemical composition and sometimes also contains minor amounts of Al and Mg. It is common in deep-sea Mn nodules and in soils, and it has been detected in concretions formed in topsoil at a trickle irrigation outlet (Chen, 1984). Its occurrence in groundwater aquifer systems has yet to be reported.

**Fe- and Mn-Oxidizing Bacteria**

The Fe- and Mn-oxidizing bacteria are a diverse group of organisms. Some (e.g., *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*) are capable of oxidizing Fe under acidophilic conditions (pH 1-4). Other Fe-oxidizing bacteria thrive at neutral pH conditions. These neutrophilic organisms are often associated with Fe-precipitates and include *Gallionella ferruginea*, *Crenothrix*, *Sphaerotilus*, *Leptothrix*, and *Metallogenium* spp. These neutrophilic organisms have been reported in groundwater seeps, water wells, and field tile drainages. Other bacteria that have been implicated in Fe- and Mn-oxidation include *Pedomicrobium*, *Hyphomicrobium*, *Clonothrix*, *Toxothrix*, and *Siderocapsa* spp. as well as magnetotactic bacteria. For some of these
organisms, the ability to oxidize Fe or Mn has been challenged (e.g., *Hyphomicrobium* spp.). A review of the genus *Pedomicrobium* is presented in Chapter IV of this document. Presently, little information is available on the genera *Crenothrix*, *Clonothrix*, *Toxothrix*, and *Siderocapsa* although these genera are still recognized (Hirsch, 1989b; 1989c; 1989d; Tuovinen et al., 1989). Although the genus *Metallogenium* has been described (Zavarzin, 1989), there is some question as to whether the *Metallogenium*-like particles formed are cellular in nature or byproducts of the metabolisms of other organisms (Miyajima, 1992).

The identification of Fe-oxidizing bacteria, particularly of *Leptothrix* and *Gallionella* spp., relies on the characteristic stalk or sheath of the organism. Experimental evidence for species identification and isolation often consists of nothing more than the microscopic observation of sheaths associated with Fe-precipitates (Emerson and Revsbech, 1994a; 1994b). Characteristic cellular assemblies can also readily be observed using electron microscopy. In general, microorganisms tend to be encrusted with precipitates in samples with elevated levels of Fe and Mn. Encrustations associated with cell walls, sheaths, and capsules can preserve the characteristic shape of the organism after cell death and lysis, as shown in Figure 3. Figure 4 shows filament- and stalk-like structures which are covered with granular precipitates while the structures retain their helical orientation. Because of the lack of pure cultures, microscopic observation has historically been the major criterion for identification. Consequently, the actual definition of a genus of Fe-oxidizers is often
Figure 3. Transmission electron micrographs of thin sections of bacteria retrieved from well water samples. The cells are surrounded by electron-dense layers which have retained the cellular shape; electron-transparent interior regions show the absence of any cytoplasmic structures due to cell lysis. Ultrastructural details of the cell wall cannot be resolved in these specimens. Bars = 500 nm
Figure 4. Electron micrographs of encrusted filaments and stalks, some resembling *Gallionella*-type structures. A, SEM-micrograph of encrusted filamentous structures, showing a hollow interior of the completely encrustation-surrounded helical structure (bar = 1 μm). B, TEM micrograph of a thin section which is taken to represent helical structures that are separated from each other in this thin-sectioned plane (bar = 1 μm).
vague and the species designations are questionable. The definitions of many of the genera of Fe- and Mn-oxidizing bacteria remain speculative at best and await confirmation with physiological, biochemical, and phylogenetic analysis of pure cultures. Current research involving members of the genera *Gallionella*, *Leptothrix*, and *Sphaerotilus* is summarized, and a brief review of those organisms is presented in the following sections.

*Gallionella*

The stalk-forming bacteria, *Gallionella ferruginea*, are described as chemolithoautotrophs, requiring CO$_2$ as a C source and Fe(II) as an energy source. Its chemolithoautotrophic nature remains in doubt for several reasons. The abiotic oxidation of Fe$^{2+}$ by dissolved O$_2$ is extremely rapid at neutral pH values and leads to the formation of Fe-encrustations characteristically found associated with *Gallionella* stalks. Most media formulations for *Gallionella* supply Fe(II) as amorphous, synthetic FeS which tends to be oxidized abiotically in oxygenated media. Lutters-Czekalla (1990) reported that *Gallionella* cells were capable of growing with only sulfide or thiosulfate as an electron donor. The S-oxidation by *Gallionella* raises doubts as to whether it is the Fe- or S-entity of FeS that the bacterium oxidizes in standard *Gallionella*-media formulations. On the other hand, it has been demonstrated that *G. ferruginea* can grow with FeCO$_3$ (siderite) as the sole electron donor (Hallbeck et al., 1993; Hallbeck and Pedersen, 1993). However, Hallbeck et al. (1993) were unable to
grow their isolate with sulfide or thiosulfate as the sole electron donor.

Historically, Wolfe's FeS medium (with FeS as the electron donor and CO$_2$ as the carbon source) is commonly used for enrichment and maintenance of *Gallionella* (Hanert, 1989). Currently, pure cultures of *Gallionella* are not available in public domain. Only one species, *G. ferruginea*, is recognized. Identification of *Gallionella* relies upon microscopic observation of twisted stalks and the ability of the organism to utilize Fe(II) as the energy source and CO$_2$ as the sole carbon source. However, growth conditions have been reported to influence the formation of the stalk. Under microaerophilic conditions with CO$_2$ as the C source and FeS as the electron donor, the organism does not form stalks (Hallbeck and Pedersen, 1990). Lütters-Czekalla (1990) reported that a *Gallionella* isolate oxidized sulfide and thiosulfate but stalk formation did not occur during the growth. Lütters-Czekalla (1990) speculated that because of Fe-encrustation the stalk formation is a by-product of Fe(II)-oxidation.

These examples suggest that *Gallionella* may be present in water samples that do not contain the characteristic stalks. Phylogenetic studies based on 16S rRNA gene sequence align *G. ferruginea* with the Proteobacteria and yield a 90% similarity to *Nitrosolobus multiformis* (Hallbeck et al., 1993). Phylogenetic analysis of other cultures have revealed a stalkless variant of *G. ferruginea* (Hallbeck et al., 1993), suggesting false-negative *Gallionella* samples if the identification relies on stalk formation alone. Based on the unique sequences in 16S rRNA, it is possible to design oligonucleotide probes that are specific for *Gallionella* spp. (Hallbeck et al., 1993).
The ultrastructure of *G. ferruginea* cells, with the stalks removed by Na-thioglycolate treatment, revealed a cell wall structure typical of Gram-negative bacteria (Lütters and Hanert, 1989). Although it is believed that CO$_2$ is assimilated via the Calvin cycle, carboxysomes have not been observed in ultrathin sections of *G. ferruginea* (Hanert, 1989; Lütters and Hanert, 1989). Lütters and Hanert (1989) speculated that *G. ferruginea* does not produce carboxysomes when CO$_2$ is not limiting, but this has yet to be experimentally confirmed. If the organism has ribulose-bisphosphatecarboxylase activity but no carboxysomes, it would be rather unique and would make an interesting host to study chemoautotrophy.

The generation time of *G. ferruginea* is 8.3 h when grown aerobically with CO$_2$ as the sole C and FeS as the electron donor (Hallbeck and Pedersen, 1990). *G. ferruginea* does not grow under anaerobic conditions or in the absence of a C source (Hallbeck and Pedersen, 1990). No growth was observed in thiosulfate media supplemented with acetate, pyruvate, fumarate, succinate, yeast extract, or peptone, suggesting obligate autotrophy and sensitivity to organic compounds (Lütters-Czekalla, 1990). In contrast, Hallbeck and Pedersen (1991) demonstrated active uptake of glucose, fructose, or sucrose by *G. ferruginea*, and 25-48% of the C from the sugars was incorporated into biomass or respired. The growth rates under mixotrophic and autotrophic conditions were comparable.

Heldal and Tumyr (1983) suggested an inorganic chemical composition of *Gallionella* stalks because they did not detect P in their partial elemental analyses;
Hanert (1989) also noted that stalks are inorganic. Other reports suggest that stalks may contain proteins (Ghiorse 1984). Hallbeck and Pedersen (1993) compared the C:N values for stalked and non-stalked *G. ferruginea* and concluded that the stalks contained a carbon skeleton. The function of the stalk remains speculative. Hallbeck and Pedersen (1993) suggested a protective role for stalks against Fe-precipitation and encrustation, and it may be a response to selective pressures because prolonged culturing of *G. ferruginea* on FeCO₃ led to a loss of stalk formation.

**Leptothrix**

*Leptothrix* spp. are rod-shaped cells that are either free-living or contained in a sheath that accumulates Mn and Fe (Ghiorse, 1984). Freeze-substitution of sheathed *L. discophora* cells indicated that the individual cells are immediately surrounded by capsular material and then contained in the sheath (Graham et al., 1991). The free-living cells generally have one polar flagellum. Adams (1985) reported that prolonged culturing of *Leptothrix* spp. selected for a strain SS1 (ATCC 43182) which was no longer capable of sheath formation. Almost all reported *Leptothrix* spp. are capable of oxidizing Mn, except for *L. discophora* 001 and the obscure *L. pseudoochracea* (Corstjens et al., 1992; Dubinina, 1978). The validity of the identification of *L. pseudoochracea* is presently doubtful. *Leptothrix* spp. are chemoorganotrophs which can utilize some sugars and organic acids as the sole C and energy sources (Mulder, 1989). Currently, five different species of *Leptothrix* are recognized: *L. ochracea*, *L.*
pseudoochracea, L. discophora, L. cholodnii, and L. lopholea (Mulder, 1989). Older reports of Sphaerotilus discophorus refer to organisms currently classified as either L. cholodnii or L. discophora (Mulder, 1989).

The oxidation of Mn is the most distinctive feature of Leptothrix spp. The Mn-oxides thus formed tend to be poorly crystalline with an average oxidation state below 3.6 (Adams and Ghiorse, 1988). The oxidation of Mn is mediated by at least two extracellular enzymes produced by Leptothrix spp. The supernatants from spent cultures of Leptothrix discophora SS-1, a strain no longer able to form a sheath, could support the oxidation of Mn (Boogerd and deVrind, 1987). The Mn-oxidation activity was heat labile, sensitive to inhibitors, and had a pH optimum, suggesting that the oxidation of Mn was enzymatic and involved two active proteins with molecular weights of 110,000 and 85,000 (Boogerd and deVrind, 1987). The 85,000 protein was a degradation product of the larger protein (Adams and Ghiorse, 1987). The presence of outer membrane “blebs” containing the Mn-oxidizing protein was also proposed (Adams and Ghiorse, 1987). The oxidation of Mn may provide little useful energy for the cells. High Mn concentrations reduced the cell yield, shortened the long-term survival of cells, and decreased the growth rate (Adams and Ghiorse, 1985). It was previously suggested (Adams and Ghiorse, 1985) that the adverse effects of Mn on L. discophora SS-1 may be due to a nutrient deprivation caused by the scavenging of nutrients through sorption to Mn-oxides.
Although commonly regarded as Mn-oxidizing bacteria, *Leptothrix* spp. are also involved in Fe-oxidation. Spent culture media of *L. discophora* SS1 catalyzed the oxidation of Mn, suggesting the production of extracellular, Mn-oxidizing enzymes (Ghiorse, 1984). A 150 kDa protein was detected in spent culture medium of *L. discophora* SS1 which was capable of catalyzing the oxidation of Fe only at pH>5.0 (Corstjens et al., 1992). A mutant strain, *L. discophora* 001, produced intracellular Mn- and Fe-oxidizing proteins but intact cells did not oxidize Fe or Mn, suggesting that Fe- and Mn-oxidizing enzymes had an extracellular physiological function (Corstjens et al., 1992).

*Leptothrix* spp. and *Sphaerotilus* spp. are sometimes grouped together, but only *Leptothrix* spp. are capable of oxidizing Mn (Corstjens et al., 1992). The oxidation state of the Mn-oxides is estimated to be 3.6 (Adams and Ghiorse, 1988), indicating only partial oxidation. It is not possible to distinguish between biotically- and abiotically-produced Mn oxides.

The sheaths of *Leptothrix* spp. have been characterized with respect to their structure and possible functions. The sheaths of *L. discophora* grown under laboratory culture conditions could only be retained if the organisms were cultured with Mn, and the loss of sheath production was not reversible (Emerson and Ghiorse, 1992). The Mn-oxidizing protein was found to be closely associated with the sheath because cell-free sheaths were capable of oxidizing Mn. The Mn-oxidizing protein was probably produced intracellularly and secreted since sheath-free mutant cells as well as non-
mutant cells were capable of oxidizing Mn (Emerson and Ghiorse, 1992; 1993a). The sheath of *L. discophora* has a polymeric structure of fibrils that are composed of heteropolysaccharide-protein complexes (Emerson and Ghiorse, 1993a; 1993b). The sheath proteins have many cysteine residues, and the sulfhydryls of the cysteine residues are available for disulfide bond formation which are integral for maintaining the rigidity of the sheath (Emerson and Ghiorse, 1993b). Because the sheath was found to be resistant to many enzymes and chemical denaturants, Emerson and Ghiorse (1993b) suggested that the sheath provided protection to the cells from various environmental stresses.

*Sphaerotilus*

The genus *Sphaerotilus* has only one recognized species, *S. natans*, and is described as rod-shaped organisms either contained within a sheath or free-living without a sheath. Sheathless cells are motile by means of subpolar flagella. *Sphaerotilus* spp. utilize several sugars, alcohols, and organic acids but do not oxidize Mn-compounds. *Sphaerotilus* spp. have been classically referred to as Fe-precipitating organisms because the sheath acts as a nucleation point for precipitation.

*Sphaerotilus* spp. are usually found in activated sludges or in biofilms on rotating biological contactors in waste water treatment systems (Kinner et al., 1983). Identification of *Sphaerotilus* spp. in biofilms or sludge has usually been by means of phase contrast microscopy, yielding generally qualitative information. Howgrave-Graham and Steyn (1988) developed a fluorescent antibody (FA) detection technique
for *Sphaerotilus* spp., with whole cells of unsheathed *S. natans* used as antigens. The FA method was relatively specific both for free-living and sheathed *S. natans* and did not cross react with six other bacterial isolates from an activated sludge sample (Howgrave-Graham and Steyn, 1988). Cross reactivity against *Leptothrix* spp. was not tested.

Some morphological similarities exist between *Sphaerotilus* spp. and *Leptothrix* spp., such as the ultrastructure and chemical composition of the sheath (Emerson and Ghiorse, 1993a), but *Sphaerotilus* spp. are not capable of Mn-oxidation. Studies involving phylogenetic analyses have not clearly classified the relationship between *Sphaerotilus* spp. and *Leptothrix* spp. Based on 5S rRNA sequencing data, *Leptothrix* spp. were not closely related to *Sphaerotilus* spp. (Willems et al., 1991) but analysis based on 16S rRNA sequencing data indicated that *Leptothrix* spp. and *Sphaerotilus* spp. were distinct genera and that both belonged to the β1-subdivision of the Proteobacteria (Corstjens and Muyzer, 1993). These data are inconclusive in classifying *Leptothrix* spp. and *Sphaerotilus* spp.

**Goals of This Investigation**

The specific goals of the investigations presented in Chapters II and III were as follows: 1. Identification of the Fe precipitates formed by heterotrophic iron-precipitating bacteria in laboratory cultures and water well systems. The precipitates, whose formation is in part biologically mediated, are involved in the biofouling of
water wells. An understanding of the mineralogical structure of these precipitates is useful in prescribing treatments for the remediation and prevention of Fe-biofouling of water wells. 2. The construction and testing of a flow-cell device for monitoring biofilms associated with Fe-precipitating microorganisms in water wells. The flow-cells were tested in both laboratory and field situations, providing a device for observing biofilms containing organisms associated with biofouling that could not be cultured in the laboratory. 3. The enrichment and partial characterization of bacteria associated with the biofilm colonization of glass microscope slides. Attempts were made to enrich for *Leptothrix* spp. and *Gallionella* spp. Isolates of methylotrophic, appendaged organisms classified as *Hyphomicrobium* spp. were obtained.
CHAPTER II

FERRIHYDRITE IN WATER WELLS AND BACTERIAL ENRICHMENT CULTURES

Abstract

The chemical and mineralogical composition of Fe(III)-precipitates formed in water wells and laboratory cultures was examined. Ferrihydrite, a poorly ordered Fe(III)-oxide of bulk formula \(5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}\), was detected by x-ray diffraction analysis in all samples. The crystallinity varied from one sample to another. Fe was the only major element detected by energy-dispersive x-ray spectrometry; several other elements were present at minor levels reflecting the chemical composition of the medium. The results suggest that the biologically-catalyzed iron encrustation of water wells begins with the initial formation of ferrihydrite.

Introduction

Metallic structures immersed in water wells often become encrusted with Fe(III) precipitates which reduce the hydraulic performance of the well and the quality of the water withdrawn. The formation of Fe(III) precipitates is typically catalyzed by
microorganisms. The deposition of Fe(III) precipitates on metal structures also sets up differential aeration cells which become focal points for corrosive attack. Iron encrustations harbor a diverse spectrum of bacteria due to aerobic and anaerobic zones in interior layers, thus further contributing to the biofouling problems.

Ochreous precipitates formed in water wells and ground water treatment plants are sometimes referred to as amorphous Fe(OH)$_3$. Iron oxides formed under these conditions vary in their crystallinity but often are poorly crystalline (Carlson and Schwertmann, 1987). The crystallinity influences many properties of iron oxides, e.g., aggregation of iron oxide particles and ability to scavenge metal ions from the surrounding water. Aggregation may lead to clogging problems in filter materials and screens. Therefore, information on mineralogical aspects of iron transformations is helpful in assessing the fate and role of iron in water supply.

For the present work, chemical and mineralogical properties of Fe(III) precipitates formed in water wells were characterized. Fe(III)-precipitates were also produced under laboratory conditions in defined, synthetic media inoculated with water well samples. X-ray diffraction (XRD), energy dispersive x-ray spectrometry (EDS), and electron microscopy were employed to document variation in mineralogical and morphological characteristics of sample materials.
Materials and Methods

Three well sites, all with histories of iron-related biofouling problems, were used as sources of samples of iron precipitates and bacterial inocula for laboratory studies. Iron encrustations from water wells were collected on glass slides immersed in well-head collectors, thereby providing a surface for microbial colonization and biofilm formation. The collectors were approximately 1.5-liter flow cells, 30 cm by 10 cm, and constructed of polycarbonate plastic (Lexan). The collectors could accommodate two slide holders, each capable of housing eight removable glass microscope slides (Smith and Tuovinen, 1990).

Water samples obtained from well-head taps were collected in sterile, 150 ml containers. These samples were used as inocula for studies involving biologically-mediated iron encrustations in a mineral salts medium supplemented with various iron sources. The mineral salts medium, a modified version of W-R medium (Cullimore and McCann, 1977), contained (per liter): K$_2$HPO$_4$, 0.5 g; MgSO$_4$•7H$_2$O, 0.5 g; CaCl$_2$•2H$_2$O, 50 mg; and either (NH$_4$)$_2$SO$_4$ or NH$_4$NO$_3$, 0.5 g. The mineral salts medium was amended with either 1, 2, or 3 mM Fe(III) ammonium citrate, Fe(III) citrate, Fe(II) gluconate, or Fe(II) choline citrate, and incubated for up to twelve weeks at 20°C (Table 2).

Liquid media inoculated with well water samples yielded iron precipitation within 2 to 7 days of incubation. The precipitation did not occur in sterile control media. Microscopic examination of active cultures revealed rod-shape bacterial cells;
Table 2. Description of experimental conditions.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Incubation Mode</th>
<th>Incubation Weeks</th>
<th>Initial pH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fe Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Shaking</td>
<td>4</td>
<td>7.0</td>
<td>2 mM Fe(III) ammonium citrate</td>
</tr>
<tr>
<td>2</td>
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<td>4</td>
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<td>2 mM Fe(III) citrate</td>
</tr>
<tr>
<td>4</td>
<td>Static</td>
<td>4</td>
<td>7.0</td>
<td>2 mM Fe(II) gluconate</td>
</tr>
<tr>
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<td>12</td>
<td>5.7</td>
<td>2 mM Fe(III) ammonium citrate</td>
</tr>
<tr>
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<td>Static</td>
<td>12</td>
<td>5.7</td>
<td>2 mM Fe(III) ammonium citrate</td>
</tr>
<tr>
<td>7</td>
<td>Static</td>
<td>12</td>
<td>5.7</td>
<td>2 mM Fe(III) ammonium citrate</td>
</tr>
<tr>
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<td>Static</td>
<td>12</td>
<td>5.7</td>
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<tr>
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<td>13</td>
<td>Static</td>
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<td>7.0</td>
<td>3 mM Fe(II) choline citrate</td>
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<td>7.0</td>
<td>1 mM Fe(II) choline citrate</td>
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<tr>
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<td>7.0</td>
<td>2 mM Fe(III) ammonium citrate</td>
</tr>
<tr>
<td>17</td>
<td>Static</td>
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<td>7.0</td>
<td>1 mM Fe(II) choline citrate</td>
</tr>
<tr>
<td>18</td>
<td>Static</td>
<td>1</td>
<td>7.0</td>
<td>3 mM Fe(II) choline citrate</td>
</tr>
<tr>
<td>19</td>
<td>Static</td>
<td>1</td>
<td>7.0</td>
<td>1 mM Fe(II) choline citrate</td>
</tr>
<tr>
<td>20</td>
<td>Well-head Collector</td>
<td></td>
<td></td>
<td>Natural well water</td>
</tr>
</tbody>
</table>

<sup>a</sup>The pH values were in the range of 7.8-8.2 after one week and 8.6-8.9 after four weeks of incubation.
sheathed and stalked forms were uncommon. All enrichment cultures precipitated iron. The Fe(III) precipitates formed during the incubation were collected by centrifugation and dried at 40°C for analysis.

X-ray diffraction (XRD) analysis of topfill powder mounts were conducted using CuKa-radiation with a Philips PW 1316/90 wide-angle goniometer equipped with a diffracted beam monochromator and a Θ compensating slit. All specimens were scanned from 10 to 70°2Θ in increments of 0.1°2Θ and with 20 seconds counting time.

Energy dispersive elemental analysis (EDS) was conducted under an atmosphere of air using an x-ray fluorescence spectrometer (Tracor X-ray Spectrace 4050). The tube voltage was 10 keV, the tube current 0.01 mA, no filter was used, and counts were collected for a livetime of 100 seconds. This technique was not calibrated for quantitative analysis.

Transmission electron microscopy was used to examine variation in the morphology of the samples used for XRD and EDS analyses. The precipitates were dried at 40°C, washed three times, and resuspended in double distilled demineralized water. The samples were placed on formvar/carbon coated copper microscope grids and examined using a Zeiss 10 electron microscope.
Results and Discussion

The number of XRD-lines as well as the position of the most intensive line can be used as indicators of the crystallinity of ferrihydrite. In the XRD-pattern of well crystalline ferrihydrite, six distinct lines are present with the d-value of the main line at 2.53 Å. With a decreasing order, all the lines weaken and broaden and those at 1.97 and 1.72 Å are the first to disappear. At the same time, the lines at 1.51 and 1.47 Å form an unresolved doublet at 1.5 Å. The next line to disappear is that at 2.2 Å and the main line shifts further to a higher d-value (Carlson and Schwertmann, 1981). The two remaining lines are brought about by two-dimensional arrangement of Fe(O,OH) octahedra. Two-line iron oxide often occurs in connection with better ordered ferrihydrite and there is a continuous transition from perfectly ordered to highly disordered structures. Therefore, in keeping with Schwertmann and Taylor (1989), the 2-line material is construed to represent poorly crystalline ferrihydrite. Of the 20 specimens studied, one contained 5-line, one 3-line, and 18 2-line ferrihydrite (Figure 5, Table 3).

The position of the main line in 2-line ferrihydrites ranged from 2.61 Å to 2.85 Å (Table 3), and with an increasing d-value of the main line, the intensity of the 1.5 Å line decreased. In most 2-line ferrihydrite samples, a faint indication of the third line at 2.2 Å was discernible. The precipitate from the well water collector (sample no. 20) clearly showed three lines. The 5-line ferrihydrite specimen (sample no. 10) contained a salt residue from the culture medium that interfered with the ferrihydrite peaks,
Figure 5. X-ray diffractograms of Fe(III)-precipitates. A, 6-line ferrihydrite (abiotic, prepared as described by Eggleton and Fitzpatrick, 1988); B, 5-line ferrihydrite (sample no. 10); C, 2-line ferrihydrite (sample no. 2); D, 2-line ferrihydrite with a plateau (sample no. 18); E, 3-line ferrihydrite (sample no. 20). Sample no. 20 was analyzed as a thin film on a glass slide which caused an increasingly high background.
Table 3. Observed d-values of XRD-lines in precipitate samples.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>d-values (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.62 1.51</td>
</tr>
<tr>
<td>2</td>
<td>2.63 1.50</td>
</tr>
<tr>
<td>3</td>
<td>2.69 1.50</td>
</tr>
<tr>
<td>4</td>
<td>2.85 1.55</td>
</tr>
<tr>
<td>5</td>
<td>2.66 1.51</td>
</tr>
<tr>
<td>6</td>
<td>2.64 1.51</td>
</tr>
<tr>
<td>7</td>
<td>2.64 1.50</td>
</tr>
<tr>
<td>8</td>
<td>2.63 1.51</td>
</tr>
<tr>
<td>9</td>
<td>2.70 1.51</td>
</tr>
<tr>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~2.55 ~1.49</td>
</tr>
<tr>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>~1.5</td>
</tr>
<tr>
<td>12</td>
<td>2.63 1.51</td>
</tr>
<tr>
<td>13</td>
<td>3.80&lt;sup&gt;sh&lt;/sup&gt; 2.61 1.51</td>
</tr>
<tr>
<td>14</td>
<td>2.72 1.53</td>
</tr>
<tr>
<td>15</td>
<td>2.63 1.51</td>
</tr>
<tr>
<td>16</td>
<td>4.20&lt;sup&gt;sh&lt;/sup&gt; 2.66 1.50</td>
</tr>
<tr>
<td>17</td>
<td>4.55&lt;sup&gt;pl&lt;/sup&gt; 2.72 1.51</td>
</tr>
<tr>
<td>18</td>
<td>4.50&lt;sup&gt;pl&lt;/sup&gt; 2.63 1.52</td>
</tr>
<tr>
<td>19</td>
<td>2.72 1.55</td>
</tr>
<tr>
<td>20</td>
<td>2.82 2.10 1.51</td>
</tr>
</tbody>
</table>

<sup>a</sup>5-Line ferrihydrite sample contained a salt residue which interfered with accurate estimates of d-values. The sample had three additional peaks as shown in Figure 5.

<sup>b</sup>2-line ferrihydrite sample contained a salt residue which interfered with accurate estimates of d-values.

<sup>c</sup>sh, shoulder; pl, plateau
making it difficult to determine the exact peak positions and changing the relative intensities. An estimate of the position of the main line is 2.55 Å. Ferrihydrite in this sample was formed under more alkaline conditions (pH 8.4) compared with the other samples (Table 2).

In XRD-patterns of four precipitate samples formed with Fe(II) choline citrate as the iron source, a shoulder or plateau existed from approximately 4.5 Å to the main peak at 2.5-2.7 Å (Figure 5). This plateau has been previously observed in XRD-patterns of natural, organic C-rich Fe(III)-precipitates (L. Carlson, unpublished results), but the reason for this effect is not known at the present time. Other Fe(III)-oxides or oxyhydroxides were not detected in these samples.

Partial qualitative elemental analysis of samples 1-19 revealed Fe as the only main element and minor amounts of P, Cl, K, Ca, Cr, and Mn. Mn and Cr are impurities and the other minor elements reflect the mineral salts of the growth media. Additionally, Si was detected in some precipitate samples from water wells.

Electron micrographs of both 2-line and 5-line ferrihydrites are presented in Figure 6. Both ferrihydrite samples consisted of large aggregates, 100-500 nm in diameter. The aggregates comprise individual spherical particles in the range of 2-5 nm (Carlson and Schwertmann, 1981, Schwertmann and Taylor, 1989). In general, these samples are comparable with previous ferrihydrite descriptions of either natural or synthetic origin.
Figure 6. Transmission electron micrographs of (A) 2-line ferrihydrite (sample no. 2); and (B) 5-line ferrihydrite (sample no. 10). Bars = 100 nm
Of the most common iron oxide minerals, lepidocrocite is formed through rapid oxidation of Fe(II); e.g., aeration of FeCl$_2$ solutions (Carlson and Schwertmann, 1990). At high rate of Fe supply, however, the formation of ferrihydrite is favored if silicate, phosphate, or organic matter are present (Schwertmann and Thalman, 1976, Schwertmann et al., 1984, 1986). Goethite, the stable and most widespread oxyhydroxide of iron, is preferentially formed at low rate of Fe supply from either Fe$^{2+}$ or Fe$^{3+}$ cations in solution. Moreover, metastable iron oxides convert via solution to goethite (Schwertmann and Taylor, 1989). In these experiments, the presence of organic compounds, and also silica in the case of the water well sample, has hindered the crystallization of FeOOH phases. As a result, these compounds have brought about the precipitation of a poorly ordered compound with a range of crystallinities. For most of these samples, the XRD-patterns indicate a three-dimensional order and the precipitates can therefore be considered as ferrihydrite. The better crystallinity of the 5-line ferrihydrite, which also has smaller line widths, may have been brought about by a slower rate of iron oxide formation or less effective inhibition by foreign compounds.

In conclusion, the results refute the existence of amorphous ferric hydroxide in biofouling samples. Iron precipitates formed in bacterial cultures and on exposed slide surfaces immersed in water wells can now be defined mineralogically. Poorly-ordered ferrihydrite forms aggregates more readily than better ordered ferrihydrite and has a smaller specific surface area (Carlson and Schwertmann, 1981). Ferrihydrite is more
effective in adsorbing cations and anions than better crystalline iron oxide minerals, which has relevance in scavenging and retention of nutrient and toxic-metal ions and in estimating mass balances and fluxes of major and minor elements to and from water. This kind of information is useful in understanding the dynamics of buildup of biofouling deposits and interactions with organisms and dissolved compounds in surrounding water.

Acknowledgements. Partial support for the work was received from the American Water Works Foundation through the S.A. Smith Consulting Services (L.T. and O.H.T.) and from the Tor and Maj Nessling Foundation (L.C.). We thank Dr. J.M. Bingham, Department of Agronomy, The Ohio State University, for use of XRD-facilities and Mr. S.A. Smith for providing the original sample materials.
CHAPTER III

MICROBIOLOGICAL ANALYSIS OF IRON-RELATED BIOFOULING IN WATER WELLS AND A FLOW-CELL APPARATUS FOR FIELD AND LABORATORY INVESTIGATIONS

Abstract

A flow-cell assembly was constructed to collect biofilm samples on glass slides in an effort to monitor Fe-precipitating microorganisms in water wells. The flow-cell was used with a once-flow-through connection to municipal water wells which had previous histories of Fe-related precipitation and biofouling problems. Microscopic observations of biofilms attached to glass slides confirmed the presence of stalked and sheathed bacteria believed to be involved in Fe(III)-precipitation. Scanning electron microscopy confirmed that biofilm samples contained Gallionella-type morphological features composed of helical, intertwined stalks. Attempts to recover these organisms with cultural methods were unsuccessful. For monitoring Fe-precipitating microorganisms, a differential solid medium was developed from the general-purpose heterotrophic plate-count medium (R2A) by incorporating ferric ammonium citrate in the formulation. Organisms capable of using citrate in the modified medium yielded
rust-colored colonies due to the formation of Fe(III)-precipitates. These Fe-
precipitating bacteria did not possess stalks, sheaths, or other appendages and their
presence could not be predicted from microscopic examination of the sample materials.
Optical measurement of biofilm thickness was deemed unreliable because of large
variation in the thickness of Fe(III)-precipitation and associated bacterial attachment.
Fe(III)-precipitates were identified as ferrihydrite, a poorly crystalline Fe(III)-oxide.
Laboratory studies with unamended well water samples recirculating through a flow-
cell were used to enrich for organisms involved in biofouling communities. These
experiments yielded methylotrophic organisms capable of growing with methanol and
methylamine and they were classified as *Hyphomicrobium* spp.

**Introduction**

Iron in groundwater has an impact on chemical and microbiological aspects of
in-situ and pumped water quality. When exposed to oxygenated conditions in water
wells, Fe$^{2+}$ is readily oxidized by molecular oxygen.

$$4\text{Fe}^{2+} + \text{O}_2 + 4\text{H}^+ \rightarrow 4\text{Fe}^{3+} + 2\text{H}_2\text{O} \quad (1)$$

In addition to the chemical, abiotic reaction, iron oxidation may involve a
microbiological mechanism. Subsequent hydrolysis produces poorly crystalline
Fe(III)-oxides which have low solubilities, with $K_{sp}$ values in the order of $10^{-40}$
(Macalady et al., 1990).
The formation of solid-phase products such as ferrihydrite ($\text{Fe}_5\text{O}_8\cdot 4\text{H}_2\text{O}$) is one of the initial events leading to biofouling problems (Carlson and Schwertmann, 1987; Tuhela et al., 1992). Only ferrihydrite has been identified as a crystalline solid-phase product of iron in biofouled water wells (Carlson and Schwertmann, 1987; Tuhela et al., 1992).

Biofouling due to Fe-precipitation poses several water quality and performance problems to users of water supply and other pumping wells. Extensive biofouling can clog well screens, pumps, and downstream pipes and filters, necessitating rehabilitation measures. Such problems may lead to corrosive conditions on surfaces of immersed metal structures. Biofouling can also result in poor water quality for the consumer and interfere with other treatment methods in water supply and groundwater remediation. Iron-related biofouling is one of the most common problems in water well maintenance and in pump-and-treat systems (Chapelle, 1993).

Fe(II)-oxidation in groundwater environments is presumed to involve microorganisms in several ways. Some heterotrophic bacteria may precipitate ferric iron via utilization of carbon from organic complexes of Fe(III). Iron precipitation occurs if chemical saturation conditions are reached as a result of a change in $\text{Fe}^{3+}$ speciation caused by biological degradation of the organic Fe-complex. Others, viz. stalked and sheathed iron bacteria such as Gallionella spp., may be able to oxidize Fe(II) on sheath surfaces although the mechanisms are not understood (Hanert, 1992).

$$5\text{Fe}^{3+} + 12\text{H}_2\text{O} \rightarrow \text{Fe}_5\text{O}_8\cdot 4\text{H}_2\text{O} + 15\text{H}^+$$ (2)
All other bacteria that use ferrous iron oxidation as the sole source of energy are organisms such as *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and are largely limited to acid environments (acid sulfate soils, acid mine waters). The occurrence of *T. ferrooxidans* in iron precipitates formed upon oxidation of groundwater under ambient surface conditions has been reported (Carlson et al., 1980) but the role of acidophiles in solution phase oxidation of Fe\(^{2+}\) in groundwater and water wells is likely to be negligible because of adverse pH conditions.

Mass growth of these stalked and sheathed iron bacteria in water wells results in extensive Fe(III)-precipitation on their sheaths and stalks. Sand filters used for iron removal accumulate stalked iron bacteria (*Gallionella* spp.) which aid in the precipitative removal of iron from groundwater (Czekalla et al., 1985; Viswanathan and Boettcher, 1990). Mass growth of these organisms in sand filters may result in clogging and reduced permeability of water through the filter material, thereby decreasing hydraulic conductivity.

Methods of routine culturing and maintenance of sheathed and stalked iron bacteria have not been developed. *Leptothrix* spp. have been described as iron- and manganese-oxidizing, sheathed bacteria and only two strains, *L. discophora* SS-1 and SP-6, are publicly available from the American Type Culture Collection (Adams and Ghiorse, 1986; Emerson and Ghiorse, 1992). Pure reference cultures are not available for *Gallionella ferruginea*, a bacterium producing helical stalks and presumed to be involved in iron oxidation (Ghiorse, 1984). Recent work with *Gallionella* has
demonstrated that the bacterium lives also as a free-swimming, flagellated cell and produces a stalk when exposed to ferrous-iron in the surrounding medium (Hallbeck and Pedersen, 1990; Lütters-Czekalla, 1990). Thiosulfate- and sulfide-dependent growth of *Gallionella* has also been reported (Lütters-Czekalla, 1990). The bacterium is mixotrophic, being able to derive cellular carbon from CO$_2$ or from organic compounds (Hallbeck and Pedersen, 1991). These observations presumably suggest that *Gallionella*-type iron-bacteria may be more widely distributed than previously recognized, although confirmatory pure culture characterization of these bacteria has yet to be carried out.

The detection and confirmation of the presence of Fe-precipitating bacteria remain problems in well maintenance and rehabilitation. Confirmation is helpful for choosing a well rehabilitation approach or signalling a need for preventive treatments. Several bacteria with distinct morphological characteristics have been implicated in water well biofouling problems (*e.g.*, *Gallionella ferruginea* and *Leptothrix* spp.), but reproducible methods of recovering, culturing, and maintaining *Gallionella* and other Fe-precipitating bacteria have not been developed.

Because the collection of samples with characteristic sheaths and stalks is important in identification, sampling methods to maximize biofilm recovery are critical. Representative sampling of iron-precipitating microorganisms is an important aspect in the study of microbial and biochemical interactions. Standard Methods for the Examination of Water and Wastewater (1992) recommends centrifugation or
filtration of pumped grab samples to concentrate particulate matter for microscopic identification. One problem with this procedure is the reliance on pumped samples. If pumping does not resuspend and detach filaments and stalks of iron-precipitating organisms in-situ, microscopic examination of the samples may yield inconclusive or falsely negative results.

The purpose of this work was to evaluate a flow-cell apparatus designed for collecting biofilm samples in water wells and laboratory systems. Attempts were also made to confirm the presence of iron-precipitating bacteria in biofilm samples. Several selective media were used to enrich for *Gallionella*, *Leptothrix*, and other native bacteria from biofouled samples. Both the field and laboratory phases of the evaluation study are reported in this paper.

**Materials and Methods**

*Flow Cell Design*

An early version of the design has been previously presented (Smith and Tuovinen, 1990). The flow-cell was cylindrical, constructed of autoclavable, 3-mm thick Lexan polycarbonate plastic material (General Electric Corp.). The polycarbonate material was chosen because it can be sterilized by autoclaving. The dimensions of the flow-cells were 300 mm (height) and 100 mm (diameter), with a total volume of approximately 1.5 liters (Figure 7A). The flow-cells had slide holder inserts which housed glass microscope slides (Figure 7B,C). The slide holder inserts were constructed of polycarbonate plastic.
Figure 7. Schematic design of the flow-cell. A. Flow-cell assembly with a spool can in-place. B. Schematic of a spool can, with external sleeve removed to show the accommodation of glass slides. C. Close-up of a spool can, showing the dimensions and perforation to improve hydrovelocity.
Flow Cell Usage

When used in the field, the flow-cells were connected to the well-head tap of a water well by Tygon tubing and the assembly contained a back-flow preventer in the influent. No attempt was made to determine the extent of leaching of metals and other contaminants from the tubing. Other tubing materials were not tested in the course of the field study.

The municipal supply wells used in the field study were located in western and southern Ohio. One of the well fields was in Ohio River Valley sand and gravel and two well fields were in Silurian dolomite. The field test sites are described in a previous report (Smith, 1992). Parallel with biofilm sampling from on-line flow-cells, water samples were analyzed for Fe, Mn, sulfide, conductivity, pH, and redox potential. The range of these parameters is summarized in Table 4. The results for each well have been previously reported (Smith, 1992).

When used in the laboratory, a peristaltic pump delivered water or medium from a reservoir through silicone tubing to the flow-cell. The laboratory system was set up in a recirculating mode (Figure 8). For the laboratory study, one flow-cell was set up with dilute medium and another flow-cell was assembled with autoclaved well water. Biofilm-covered slides and well water were placed into flow-cells as inoculum. In addition, a static flow-cell was assembled which contained Wolfe’s modified FeS medium (Hanert, 1981) in order to enrich for Gallionella spp.
Table 4. Range of physical and chemical parameters of water quality in three municipal water wells monitored in this study (Smith, 1992).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Fe</td>
<td>0.1 - 3.0 mg/l</td>
</tr>
<tr>
<td>Total Mn</td>
<td>&lt;0.01 - 0.7 mg/l</td>
</tr>
<tr>
<td>Total sulfide</td>
<td>&lt;0.05 - 1.1 mg/l</td>
</tr>
<tr>
<td>Conductivity</td>
<td>390 - 1400 µmhos/cm</td>
</tr>
<tr>
<td>pH</td>
<td>6.0 - 8.4</td>
</tr>
<tr>
<td>Redox potential</td>
<td>-25 - +380 mV</td>
</tr>
</tbody>
</table>

\(^{a}\)The analytical methodology has been described in a previous report (Smith, 1992).

\(^{b}\)The redox potential is referenced to the standard hydrogen scale.
Figure 8. Laboratory assembly of the flow-cell apparatus. Two flow-cells were set up in a recirulating mode. A peristaltic pump was used to regulate the flow rate through the flow cell. Silicone tubing was used to connect the flow cells to the media reservoirs.
Glass microscope slides were periodically removed from the flow-cells and the biofilms on the slides were examined under phase-contrast microscopy. The thickness of biofilms on glass slides was estimated using a microscopic method described by Bakke and Olsson (1986). The mineralogical and partial elemental composition of Fe-precipitates was analyzed by X-ray diffraction (XRD) and by energy dispersive analysis of x-rays (EDS), respectively, as previously described (Tuhela et al., 1992).

Culture Media for Microbiological Studies

Iron-precipitating bacteria were enriched using a modified W-R medium (Cullimore and McCann, 1977) which consisted of (per liter) NH4NO3, 0.5 g; K2HPO4, 0.5 g; MgSO4·7H2O, 0.5 g; CaCl2·2H2O, 50 mg; and ferric ammonium citrate, 6.0 g, pH 7.0. All cultures were incubated at 22±2°C. Growth in this medium resulted in the precipitation of Fe(III) because of citrate utilization by the bacteria. In order to differentiate between iron-precipitating and other bacteria, a heterotrophic plate count medium, R2A (Reasoner and Geldreich, 1985), was supplemented with 6.0 g ferric ammonium citrate/liter. The modified R2A was designated as FeR2A medium.

A ferrous sulfide medium was used in attempts to recover *G. ferruginea* from well water and biofilm samples. This medium has FeS as the energy source and CO2 as the C source (Standard Methods for the Examination of Water and Wastewater, 1992). In addition, a thiosulfate-containing medium, with CO2 as the C source (Lütters-Czekalla, 1990), was used for *Gallionella* enrichment.
Sessile, appendaged organisms were often present on biofilm-covered microscope slides. On the basis of their morphological characteristics, they were suspected to be *Hyphomicrobium*, *Pedomicrobium*, or *Caulobacter* spp.

*Hyphomicrobium* spp. are methylotrophs (C\textsubscript{1}-utilizers), whereas *Pedomicrobium* and *Caulobacter* spp. utilize more diverse carbon substrates (Hirsch, 1989, Gebers, 1989, Poindexter, 1989). Initial attempts were made to enrich for the methylotrophic *Hyphomicrobium* spp. in laboratory flow-cells using well water amended with 0.5\% (v/v) filter-sterilized methanol as the substrate for carbon and energy. The flow-cells were inoculated with well water and field biofilm samples. Biofilms were also used as inocula for *Hyphomicrobium* enrichment in a synthetic methylotrophic enrichment medium consisting of (per liter) KH\textsubscript{2}PO\textsubscript{4}, 1.36 g; Na\textsubscript{2}HPO\textsubscript{4}, 2.13 g; MgSO\textsubscript{4}•7H\textsubscript{2}O; 0.3 g; (NH)\textsubscript{2}SO\textsubscript{4}, 0.5 g; CaCl\textsubscript{2}•2H\textsubscript{2}O, 1.99 mg; FeSO\textsubscript{4}•7H\textsubscript{2}O, 1.0 mg; MnSO\textsubscript{4}•H\textsubscript{2}O, 0.35 mg; Na\textsubscript{2}MoO\textsubscript{4}•2H\textsubscript{2}O, 0.5 mg; and supplemented with 0.5\% (w/v) methylamine•HCl or 0.5\% (v/v) filter-sterilized methanol (Hirsch, 1989). The medium was supplemented with MnSO\textsubscript{4}•H\textsubscript{2}O (50 mg/l) for manganese oxidation tests. Methylamine was replaced by 10 mM glucose or succinate in additional substrate utilization experiments. Trypticase soy agar (TSA) was used as a non-selective, general purpose medium.
Scanning Electron Microscopy

Biofilm samples on glass slides from water well flow-cells were also retrieved for scanning electron microscopy (SEM). Samples were fixed in 4% (v/v) glutaraldehyde for one hour, dehydrated in an ethanol/water series (30%, 50%, 70%, 95%, and 100% ethanol) and critical-point dried using liquid CO₂. The samples were gold coated and examined under a Hitachi S500 scanning electron microscope at 20 kV.

Results and Discussion

Biofilm Measurement

Biofilm measurements made by the method of Bakke and Olsson (1986) were found to be unreliable because the measurement were subject to many errors. For example, stalked and sheathed bacteria seemed to be attached at single points, allowing the organisms to waver at the unattached points, thus making the biofilm appear thick. Conversely, biofilms with many bacteria were often covered by precipitates which, based on energy dispersive spectroscopy (EDS) analysis, contained Fe as the main constituent. The iron precipitates restricted the movement of bacterial sheaths and stalks, making the biofilm appear thin. These observations were in direct conflict with the microscopic estimation of biofilm thickness as an indicator of biofouling and well water quality. The use of the microscopic method of biofilm thickness measurement was, therefore, abandoned.
Recovery of Microorganisms from Flow-Cells

Within 1-4 weeks of exposure in field flow-cells, rust-colored flocculation and precipitation appeared on glass slides. The presence of characteristic *Leptothrix* and *Gallionella* forms, other microorganisms, and precipitates were noted upon initial microscopic examination of the biofilm-covered slides (Figure 9). Stalked and sheathed bacteria were identified presumptively by their characteristic morphology under phase contrast microscopy. Characteristic *Gallionella* stalks and *Leptothrix* sheaths were commonly observed in field biofilm samples in association with precipitates which were determined to be composed of Fe(III)-oxides. Qualitative elemental analysis by EDS revealed that Fe was the main component in the precipitates. The x-ray diffraction pattern of a slide sample from a field flow-cell revealed that iron precipitates were poorly crystalline Fe(III)-oxide, ferrihydrite (Tuhela et al., 1992).

In the laboratory flow-cell system, recirculating dilute (10% v/v) W-R medium, Fe(III)-precipitation formed after one week of operation. With samples plated on W-R and FeR2A agar media, several iron-precipitating and non-precipitating colonies were recovered from the flow-cell effluent and the respective biofilm samples. These isolates were capable of utilizing citrate and produced orange-colored colonies on both W-R medium and FeR2A medium because of the concurrent precipitation of Fe(III) (initially available as ferric ammonium citrate). Organisms not capable of utilizing
Figure 9. Sheathed and stalked bacteria under phase contrast microscopy retrieved from a field flow-cell biofilm. The dense material (arrow) was rust-colored, indicating Fe(III)-deposition within the biofilm. Bars: A: 10 μm; B: 3 μm. Figure 9B is reprinted from Methods for Monitoring Iron and Manganese Biofouling in Water Wells, by permission. Copyright © 1992, American Water Works Association.
citrate produced colonies that were not orange in color on FeR2A medium and were not capable of growth on W-R medium.

Enrichment cultures in Fe-amended W-R medium produced precipitates which were composed exclusively of poorly crystalline (2-line) ferrihydrite (Figure 10). All three Fe-sources, ferrous choline citrate, ferric ammonium citrate, and ferric citrate, produced ferrihydrite with similar diffraction bands under these conditions. All three samples had an unresolved doublet at 1.5 Å and another broad band at 2.63-2.69 Å (Figure 10). The formation of poorly crystalline ferrihydrite was in keeping with previous XRD analysis of Fe-precipitates formed in W-R medium (Tuhela et al., 1992). The role of bacteria in iron precipitation was indirect as they changed iron speciation and saturation conditions by degrading the soluble iron complex.

Iron precipitation was absent in flow-cells recirculating biofilm-inoculated well water only. *Gallionella* stalks and *Leptothrix* sheaths were not observed on slides retrieved from the laboratory flow-cells. *Gallionella* and other stalked or sheathed organisms were not recovered on either W-R or FeR2A media. In addition, attempts to recover *Gallionella* spp. with thiosulfate and ferrous sulfide media were also unsuccessful. Sessile, appendaged organisms were, however, present on all slides in the laboratory flow-cell recirculating well water, as also was found with many biofilm samples from field flow-cells.
Figure 10. X-ray analysis of precipitates from enrichment cultures of iron-precipitating bacteria. The media contained the following Fe-sources: A, ferrous choline citrate (sampled after 3 months of incubation); B, ferric ammonium citrate (after 3 months); C, ferric citrate (after 4 weeks).
Enrichment of Sessile, Appendaged Organisms

Methylotrophic enrichment medium supplemented with methanol was inoculated with biofilm-covered slides containing sessile, appendaged bacteria. The enrichments yielded organisms which were capable of using methylamine and methanol as the sole source of carbon and energy. These isolates were not capable of oxidizing manganese, suggesting that they did not belong to the genus Pedomicrobium (Hirsch, 1989; Gebers, 1989). The isolates did not grow on TSA, other general purpose media, or with either glucose or succinate, suggesting that they did not belong to the genus Caulobacter (Poindexter, 1989). Therefore, based on their characteristic morphology and restricted substrate utilization, the sessile, appendaged isolates recovered in methylotrophic enrichment cultures were classified as Hyphomicrobium spp. Hyphomicrobium cells tended to form clusters (Figure 11) which may be a contributing factor in colonization and biofilm development.

Scanning Electron Microscopy

Scanning electron microscopic observations of biofilm-covered slides revealed helical stalks characteristic of Gallionella as well as uncoiled filamentous structures and single, rod-shaped bacterial cells (Figure 12). The uncoiled filamentous structures may be untwisted Gallionella stalks or they may be formed by organisms other than Gallionella. The filamentous structures appeared to form an interlocking network which would contribute to biofouling effects by trapping debris and other bacteria in
Figure 11. Photomicrograph (phase-contrast) of appendaged organisms enriched from biofilm-covered slides. The organisms were subsequently identified as *Hyphomicrobium* spp. Bar: 3 μm.
Figure 12. Scanning electron micrograph of a glass slide covered with stalked organisms. The sample was retrieved from a field flow-cell after four weeks exposure. Bar: 2 μm.
the biofilm. The stalked and filamentous microbial community may also act to increase surface area to nucleate Fe(III)-precipitation.

Conclusion

The flow-cell slide collectors proved versatile for field use and biofilm collection could be duplicated under laboratory conditions. The flow-cell method can be used to provide samples of intact biofilms that reflect the microbial community in the well bore. The basic flow-cell design makes the assembly suitable for a number of applications which require on-line monitoring for biofilm accumulation. Besides glass slides, other materials (e.g., plastic beads, metal coupons) may be used in the spool can under various static or flow conditions. Glass slides are preferred if samples are examined microscopically for morphologically distinct Fe-precipitating microorganisms, which are indicative of biofouling potential. Biofilms also avail themselves for studies of geochemical and contaminant transformations in aquifers.

Defined culture media were successful in recovering methylotrophs and heterotrophic iron-precipitating bacteria. Morphologically distinct, stalked and sheathed organisms were not recovered by cultural methods, although stalked and sheathed bacteria were readily observed by microscopy in samples from field flow-cell systems. Cultural methods and media formulations for Gallionella recovery were deemed inadequate. Microscopy has been used extensively to identify and enumerate iron bacteria in field situations (Czekalla et al., 1985) but it provides little insight in the
dynamic features of iron biofouling. The development of cultural recovery methods, *i.e.*, growth media for *Leptothrix*- and *Gallionella*-types of bacteria, is needed to explore chemical and biological factors interacting in iron precipitation processes that lead into biofouling phenomena.

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CHAPTER IV

HYPHOMICROBIUM SPP. AND OTHER APPENDAGED ORGANISMS
AND THE
FLAGELLAR STRUCTURE OF CAULOBACTER

Abstract

Several appendaged or dimorphic prosthecate bacteria have been previously
described including *Hyphomicrobium* spp., *Pedomicrobium* spp., *Hyphomonas* spp.,
and *Caulobacter* spp. All undergo a morphologically differential lifecycle beginning
with nascent, swarmer cells. The flagella are eventually shed and an appendage begins
to form. A cell is mature after appendage elongation is complete and daughter cells can
be produced. The method of reproduction varies among the genera but all result in a
motile, swarmer cell. Recent phylogenetic analysis of the four groups of organisms has
indicated these four genera. Of the four, *Caulobacter* is the best studied. The flagella
of *Caulobacter* spp. are composed of three different flagellin proteins whose production
is temporally and spatially regulated.
Introduction

Biofilms obtained from the water wells yielded appendaged organisms attached to slide samples obtained from many sites. An attempt was made to enrich for and isolate these appendaged organisms. One of the isolates thus obtained was characterized and classified as a *Hyphomicrobium* sp. after comparison with the morphological and metabolic characteristics of other appendaged organisms or dimorphic prosthicate bacteria (DPB) (Poindexter, 1992), such as *Caulobacter*, *Pedomicrobium*, and *Hyphomonas*. Prostheca are defined as cell-wall limited appendages that form a narrow extension of some procaryotic cells. A brief overview is presented on these organisms.

Because the subsequent experimental work was focused on motility, the second part of this introduction will present a review of the flagellar structure and motility of DPB. *Caulobacter* is the only model of an appendaged organism previously reported for motility studies. To date, the motility and chemotaxis of *Hyphomicrobium* have not been addressed in the literature.

**HYPHOMICROBIUM SPP. AND OTHER APPENDAGED ORGANISMS**

*Pedomicrobium*

Of the appendaged organisms, *Pedomicrobium* ssp. are probably the least studied. The genera *Pedomicrobium* and *Hyphomicrobium* were previously used almost interchangeably. The confusion between the two genera was due to similar
natural environments that both types of organisms inhabit (Gebers, 1989; Poindexter, 1992; Akhtar and Kelso, 1993) and a morphologically comparable lifecycle whereby motile, nascent, swarmer cells shed their flagella, develop an appendage, and produce daughter cells from the distal end of the appendage (Gebers, 1989). *Pedomicrobium* spp. usually form more than one appendage apparently at random positions along the cell body (Gebers, 1989; Poindexter, 1992). Two important physiological differences that distinguish *Pedomicrobium* spp. from *Hyphomicrobium* spp. are the inability of *Pedomicrobium* spp. to utilize methanol as the sole carbon and energy source (Sly et al., 1988) and ability of *Pedomicrobium* spp. to oxidize Mn (Gebers, 1981; Gebers, 1989). Several *Pedomicrobium* strains exist in pure culture and are available through the American Type Culture Collection (ATCC). The mechanism of Mn oxidation by *Pedomicrobium* spp. has yet to be elucidated. In this respect, comparison of *Pedomicrobium* with *Leptothrix discophora* (Adams and Ghiorse, 1987; Boogerd and deVrind, 1987) would be of interest.

Phylogenetic analysis of *Pedomicrobium* spp. has confirmed the validity of the species. According to 16S rRNA sequencing, *Pedomicrobium* and *Hyphomicrobium* are very closely related (Nikitin et al., 1990; Stackebrandt et al., 1988), but based on chemotaxonomic characteristics, the genus *Pedomicrobium* should be retained (Sittig and Hirsch, 1992).
**Hyphomonas**

*Hyphomonas* spp. undergo a morphologically differential lifecycle and reproduce from the distal end of the appendage (Moore and Weiner, 1989).

*Hyphomonas* spp. have been referred to as "marine *Hyphomicrobium*" (Moore and Weiner, 1989; Poindexter, 1992) even though *Hyphomonas* spp. are not methylotrophs and cannot utilize methanol. Because of this classification, many organisms such as the marine isolate *Hyphomonas neptunium* were regarded as *Hyphomicrobium* spp. until the results of Moore et al. (1984) and Gebers et al. (1984) justified the genus *Hyphomonas* on the basis of substrate utilization and DNA-DNA homology. Moore et al. (1984) suggested that the organism known previously as *Hyphomicrobium neptunium* be reclassified as *Hyphomonas neptunium*. The five accepted *Hyphomonas* species are *H. neptunium, H. polymorpha, H. oceanitis, H. hirschiana,* and *H. jannaschiana* (Weiner et al., 1985; Moore and Weiner, 1989). Although the work reported on the cell cycle of *Hyphomonas* spp. was published prior to the reclassification of *Hyphomicrobium neptunium* as *Hyphomonas neptunium*, the current convention of nomenclature is adapted for this review.

In initial studies of *Hyphomonas neptunium*, the cells were treated with nalidixic acid (NA) in order to inhibit DNA synthesis and replication (Weiner and Blackman, 1973). The NA-treated cells formed long appendages, and non-appendaged swarmer cells shed their flagella and also formed long appendages (Weiner and Blackman, 1973). The formation of daughter cells was dependent on DNA replication.
The possibility was not excluded that bud formation was also dependent on DNA replication. In synchronous cultures, *Hyphomonas neptunium* began to form hyphae after 85 minutes, production of daughter cell buds began after 180 minutes, and daughter cell detachment occurred after 265 minutes (Wali et al., 1980). Emala and Weiner (1983) reported that the adenylate energy charge was among the lowest in swarmer cells compared to other morphological cell types, possibly indicating that the low energy charge prevents the swarmer cells from developing into appendaged cells until reaching an optimal environment (Emala and Weiner, 1983).

The lipopolysaccharide (LPS) composition of *Hyphomonas neptunium* and *H. jannaschiana* lacks ladder-like LPS arrangements on sodium dodecyl sulfate-polyacrylamide gels (Sledjeski and Weiner, 1991). As in most other marine bacteria, an R-type LPS was present (Sledjeski and Weiner, 1991).

*Hyphomicrobium*

The methylotrophic metabolism of *Hyphomicrobium* spp. has been well characterized (Anthony, 1982). *Hyphomicrobium* spp. assimilate carbon as formaldehyde via the serine pathway (Anthony, 1982). The enzyme serine hydroxymethyltransferase (SHMT) is responsible for the incorporation of formaldehyde by catalyzing the reversible condensation of formaldehyde and glycine to form serine. Miyata et al. (1993) cloned the gene for SHMT from *Hyphomicrobium methyllovorum* GM2 into *Escherichia coli*, and demonstrated that the SHMT enzyme
from *H. methylovorum* GM2 was similar to that of *Bradyrhizobium japonicum*. An uptake system for methylamine has also been demonstrated for *Hyphomicrobium* (Brooke and Attwood, 1984). While generally classified as a facultative methylotroph (Hirsch, 1989a), several strains of *Hyphomicrobium* have been described as obligate methylotrophs (Izumi et al., 1982; Suylen and Kuenen, 1986).

Methylated sulfur compounds such as dimethyl sulfide, dimethyl sulfoxide, and methanethiol can be utilized by some *Hyphomicrobium* spp. (Suylen and Kuenen, 1986; Suylen et al., 1987; Zhang et al., 1991). Ghisalba et al. (1985) utilized the ability of a monomethyl sulfate-utilizing *Hyphomicrobium* sp. in a pilot treatment system for chemical waste material (Ghisalba et al., 1985). Dichloromethane can be utilized as the sole carbon and energy source by *Hyphomicrobium* DM2 (Stucki et al., 1981; Kohler-Staub, 1983). Henrysson and Mattiasson (1993) described a microbial biosensor system for dichloromethane measurement in waste waters, based on *Hyphomicrobium* DM2 immobilized in alginate. *Hyphomicrobium* GJ21, which was isolated from a biological trickling filter system, was capable of degrading dichloromethane in solutions containing up to 350 mM NaCl (Diks et al., 1994).

Investigation of the *Hyphomicrobium* cell cycle has necessitated the use of synchronous cultures. By using differential centrifugation and filtration to obtain synchronous cultures, Moore and Hirsch (1973) determined that *Hyphomicrobium* B-522 had an average doubling time (g) of 14.3 h and that swarmer cells began developing hyphae an average of 5 h after cell division (Moore and Hirsch, 1973).
Another method to obtain synchronous cultures is based on continuous culture of *Hyphomicrobium* B-522 in a chemostat (Matzen and Hirsch, 1982b). Chemostat cultures of *Hyphomicrobium* have also been used with C and N limitation (Gräzer-Lampart et al., 1986). The different phases of the cell cycle may influence the survival of *Hyphomicrobium* spp. in the environment. For instance, a bacteriophage was isolated from raw sewage that was capable of adsorption to cells of *Hyphomicrobium* sp. WI-926 at all stages of development but was capable of replicating only in swarmer cells (Preissner et al., 1988). Infected swarmer cells were incapable of forming appendages and thus, were not able to mature and reproduce (Preissner et al., 1988).

*Caulobacter*

Of the appendaged bacteria, *Caulobacter* are the best characterized and studied for the temporal control of the cell cycle. As with the other appendaged bacteria, *Caulobacter* spp. undergo a morphologically differential lifecycle. Nascent swarmer cells eventually shed their flagella, produce an appendage, and are able to replicate (Shapiro, 1985; Poindexter, 1989). Mature, appendaged cells have a holdfast at the distal end to anchor the cell to surfaces. Appendaged cells form daughter cells from the cell body instead of the distal end of the appendage (Shapiro, 1985).

The ultrastructural features of *Caulobacter* spp. have been examined under electron microscopy (Graham et al., 1991). Swarmer cells of *Caulobacter* spp. have pili which may help the organism attach to surfaces (Poindexter, 1981). Specific phage
receptor sites have been identified on *Caulobacter* cells which may also assist in the adsorption or attachment of the organism (Poindexter, 1981). *Caulobacter* spp. have been isolated from freshwater, marine water, soils, and wastewater treatment systems (Poindexter, 1981; Anast and Smit, 1988; MacRae and Smit, 1991). The caulobacters utilize many carbon sources, but only *C. crescentus* can grow with methanol as a carbon source (Poindexter, 1989).

The temporal regulation of the *Caulobacter* cell cycle has been studied in detail, aided in part by the development of methods for obtaining synchronous cultures (Evinger and Agabian, 1977; Swoboda and Dow, 1979). The morphological differentiation events have been reviewed previously (Newton and Ohta, 1990). The regulation and temporal control of the cell cycle events continue to be an area of research interest. Through the use of temperature sensitive mutants, Osley and Newton (1980) proposed that cell division is dependent upon DNA elongation and the completion of DNA replication. Furthermore, the replication of DNA was proposed to be a timing mechanism for cell division. The promoter region which controls the dependence of cellular differentiation on DNA replication has been characterized (Stephens and Shapiro, 1993). Protein translocation appears to have a role in cellular differentiation. A *sec A* mutant of *Caulobacter crescentus* was unable to initiate elongation of an appendage or complete DNA replication. Cells were able to initiate DNA replication and prepare for appendage elongation by shedding the flagellum and degrading the chemoreceptors (Kang and Shapiro, 1994). Kang and Shapiro (1994)
suggested that the correct translocation of proteins is necessary for completion of the cell cycle.

THE FLAGELLAR STRUCTURE OF *CAULOBACTER*

Although the appendaged organisms described previously in this chapter undergo differential lifecycles which include a motile, swarmer cell stage, only studies involving the motility of *Caulobacter* spp. have been reported. Other appendaged organisms may not have the same flagellar structure or chemotactic responses as those of *Caulobacter* spp. These characteristics in *Caulobacter* spp. may constitute an appropriate initial model of chemotaxis and reference for studies of the motility and flagellar structure of other appendaged organisms.

The flagellar structure of *Caulobacter* spp. (Figure 13) is similar to that of enteric organisms (Kornacker and Newton, 1994). A major difference in *Caulobacter* is in the basal body which contains an E-ring situated between the P-ring and M and S rings as illustrated in Figure 13 (Brun et al., 1994; Kornacker and Newton, 1994). The hook, composed of a 70 $M_r$ protein, is the next structure to form and is attached to the basal body (Johnson et al., 1979). Hook proteins are expressed only during the assembly of the flagella, and secretion of the hook protein only occurs when the basal body is present (Kornacker and Newton, 1994). The amino acid sequence necessary for export of hook proteins has been determined, and export of the hook protein probably occurs through a channel in the basal body (Kornacker and Newton, 1994).
Figure 13. The flagellar structure of *Caulobacter*. (Modified from Brun et al., 1994; Kornacker and Newton, 1994).
The flagella of most procaryotes are composed of only a single flagellin protein but some notable exceptions have been reported. *Halobacterium halobium* filaments contain five flagellins and *Helicobacter pylori* filaments contain two flagellins (Kostrzynska et al., 1991; Gerl and Sumper, 1988). *Rhizobium meliloti* filaments are assembled with heterodimers composed of two flagellin proteins (Pleier and Schmitt, 1989). *Pseudomonas aeruginosa* flagellar filaments are composed of two subgroup types of flagellin that undergo *in vivo* modification (Allison et al., 1985; South et al., 1994). *P. aeruginosa* flagella appear to consist of only one type of the two flagellins (Totten and Lory, 1990). *Salmonella* spp. have two flagellin genes whose expression is controlled by a phase variation mechanism (Zieg et al., 1977). The structure of the *Caulobacter* flagellar filament is also exceptional because it consists of at least three flagellin proteins of 29,000 $M_r$, 27,000 $M_r$, and 25,000 $M_r$ (Driks et al., 1989; Newton and Ohta, 1990). Initially, only two flagellin proteins (27,000 $M_r$ and 25,000 $M_r$) were found in *Caulobacter* flagella (Lagenaur and Agabian, 1978; Poindexter, 1981). Further studies indicated that the 29,000 $M_r$ protein was also a component of *Caulobacter* flagella (Gill and Agabian, 1983). Johnson et al. (1983) reported that a 22,000 $M_r$ protein was detected in mutants which lacked normal flagella. This protein may be a breakdown product of the 27,000 $M_r$ and 25,000 $M_r$ proteins (Johnson et al., 1983).

Schoenlein et al. (1992) suggested that the 22,000 $M_r$ protein may be a precursor for the larger flagellin proteins (Schoenlein et al., 1992). The 29,000 $M_r$
27,000 \( M_r \), and 25,000 \( M_r \) flagellin proteins are encoded on separate genes, and synthesis is regulated at the transcriptional level (Minnich and Newton, 1987; Schoenlein et al., 1992; Weissborn et al., 1982). Replication of DNA is also linked to the synthesis of the flagellin because these proteins are synthesized at the greatest rate after the replication of the DNA (Osley et al., 1977). However, the synthesis of the 25,000 \( M_r \) protein continued in the swarmer cell after separation from the appendaged cell, whereas the synthesis of the 27,000 \( M_r \) protein ceased (Osley et al., 1977). Because the mRNA transcript for the 25,000 \( M_r \) protein has a longer half-life than that of the mRNA transcript for the 27,000 \( M_r \) protein, the synthesis of the 25,000 \( M_r \) protein is likely to continue in the swarmer cells (Osley et al., 1977).

Normally *Caulobacter* flagella have all three flagellin proteins (Driks et al., 1989). Reserve pools of flagellin proteins in the cell membrane and cytoplasm of *Caulobacter crescentus* have been implicated in the assembly of the flagellar filament (Huguenel and Newton, 1984a). Because formation of the flagellum always begins at the pole opposite the appendage, the mechanism used for spatial localization of the flagellin proteins becomes a question. Huguenel and Newton (1984b) determined that both polar and non-polar vesicles were associated with *C. crescentus*, and that the reserve pool of flagellin was located in the polar vesicles. These vesicles may be involved in the translocation of proteins to specific sites for morphological changes.

The distribution of these proteins is not random. After the hook proteins, a short length of the filament is composed of the 29,000 \( M_r \) protein, followed by a longer
section of filament of the 25,000 $M_r$ protein at the distal end, with a terminal segment composed primarily of the 25,000 $M_r$ protein (Driks et al., 1989). Mutant strains have been described which have flagella composed of only the 25,000 $M_r$ protein, both the 27,000 $M_r$ and the 25,000 $M_r$ proteins, or both the 27,000 $M_r$ and the 29,000 $M_r$ proteins (Driks et al., 1989). These mutants have yet to be characterized.

The filament of a *Caulobacter* flagellum forms a right-handed helical twist, and because of this conformation the flagella rotate in a clockwise direction (Koyasu and Shirakihara, 1984). Examination of the flagella by electron microscopy indicated that *Caulobacter crescentus* flagella are surrounded by a sheath (Trachtenberg and DeRosier, 1992). The sheath may be composed of protofilaments of the flagellin proteins. Trachtenberg and DeRosier (1992) detected the presence of a 24 $M_r$ protein in the sheath, but the function of the sheath is not known.

*Caulobacter* spp. exhibit positive chemotactic responses to glucose, maltose, galactose, xylose, ribose, alanine, proline, and glutamine (Shapiro, 1985). *Caulobacter* spp. are chemotactic for only a part of the lifecycle. Proteins associated with chemotaxis such as methyl-accepting chemotaxis proteins (MCP), methyltransferase, and methylesterase are all synthesized in the predivisional cell (Gomes and Shapiro, 1984). The proteins are translocated to the swarmer cells before division and spatially oriented within the swarmer cells (Gomes and Shapiro, 1984). Nathan et al. (1986) showed that MCP were positioned in the predivisional swarmer cell and not in the predivisional appendaged cell. The MCP in the predivisional swarmer cell were not
specifically located at the flagellar pole (Nathan et al., 1986). The MCP in the nascent swarmer cell were spatially located near the flagellar pole (Alley et al., 1992).

The carboxy termini of MCP from *C. crescentus* and *E. coli* are highly conserved. The MCP from *E. coli* were expressed in *C. crescentus* and partitioned to the predivisional swarmer cell (Alley et al., 1992). Once *Caulobacter* spp. shed their flagella, the activities of MCP, methyltransferase, and methylesterase ceased (Gomes and Shapiro, 1984). As the swarmer cell differentiated into an appendaged cell, the chemoreceptor McpA was degraded (Alley et al., 1993). The proteolysis of McpA was dependent on an amino acid sequence at the carboxyl terminus of the protein (Alley et al., 1993). The protease is either cytoplasmic- or inner membrane-associated because the McpA carboxyl terminus is in the cytoplasm (Alley et al., 1993).

The understanding of the mechanisms associated with the spatial distribution and temporal regulation of chemotaxis-associated proteins and flagellar components of *Caulobacter* spp. is a reasonable starting point in the investigation of similar mechanisms in other appendaged organisms. The similarity of the cell cycle makes the *Caulobacter* model a reasonable choice with which to compare other appendaged bacteria. The production of nascent, swarmer cells from the distal end of the appendage of *Hyphomicrobium*, *Hyphomonas*, and *Pedomicrobium* poses interesting problems on the spatial distribution of flagellar components and chemotaxis-associated proteins in these cells.
GOALS OF THIS INVESTIGATION

Chapters V and VI had three major objectives as follows: 1. Enrichment and isolation of sessile, appended organisms present in biofilm samples from water wells using methylotrophic (C-1) media. Isolates having colonial and cellular characteristics similar to those of *Hyphomicrobium* spp. were obtained. Their identification as *Hyphomicrobium* was briefly confirmed. One isolate, W1-1B, was selected for a more extensive physiological and biochemical characterization. 2. Characterization of the lifecycle of *Hyphomicrobium* spp., with emphasis on W1-1B. The morphologically distinct lifecycle of *Hyphomicrobium* spp. has not yet been characterized in depth. Only few reports on the motility of the swarmer cells have been published. 3. Characterization of the chemotactic response and motility of isolate W1-1B. The structure of the flagella was also of interest because flagella are vital to the motility of *Hyphomicrobium* spp.
CHAPTER V

THE ISOLATION AND CHARACTERIZATION OF A HYPHOMICROBIUM SP.

Abstract

Biofilm and water samples retrieved from water wells were used as inocula in methylotrophic enrichment cultures. From these enrichments, 33 isolates of appendaged, methylotrophic bacteria were obtained. One isolate, designated as W1-1B, was further characterized. This isolate was an obligate methylotroph and capable of utilizing methylamine, dimethylamine, trimethylamine, or methanol as the sole carbon and energy source. The isolate was unable to grow anaerobically using nitrate as the final electron acceptor. When grown with methylamine, the doubling time (\(g\)) of W1-1B was 22.2 hours and the specific growth rate constant (\(\mu\)) was 0.031 hr\(^{-1}\). The isolate exhibited hydroxypyruvate reductase activity, indicating carbon assimilation via the serine pathway. Fatty acid profiling indicated the predominance of 18:1 cis fatty acids. These physiological data and the morphologically distinct lifecycle, examined with transmission and scanning electron microscopy, support the classification of isolate W1-1B as a *Hyphomicrobium* spp.
Introduction

The genus *Hyphomicrobium* encompasses a group of methylotrophic bacteria that are generally found in soil or aquatic environments and have been retrieved previously from biofilm communities (Hirsch, 1989; Banks and Bryers, 1991; Poindexter, 1992). In a survey of 12 strains of *Hyphomicrobium* spp. tested, none were pathogenic when injected either intravenously or subcutaneously into test animals (Famurewa et al., 1983).

*Hyphomicrobium* spp. undergo a differential lifecycle (Harder and Attwood, 1978) similar to that of *Caulobacter* (Shapiro, 1985). Initially, the nascent or swarmer cells are rod-shaped and motile by means of a single, polar flagellum. Eventually, the flagellum is shed and a stalk begins to elongate from the cell body. A cell with a stalk is considered mature and is capable of producing a daughter cell from the distal end of the stalk (Harder and Attwood, 1978).

Among the appendaged organisms, *Hyphomicrobium* spp. have been previously confused with *Pedomicrobium* and *Hyphomonas*. *Pedomicrobium* spp. can be distinguished from *Hyphomicrobium* spp. (i) by the ability to oxidize manganese and (ii) by the lack of utilization of methanol as the sole carbon and energy source (Gebers, 1981; Sly et al., 1988). Phylogenetic studies using 16S ribosomal RNA suggest that *Pedomicrobium* and *Hyphomicrobium* are related (Stackebrandt et al., 1988). *Hyphomonas* spp. are distinguished from *Hyphomicrobium* spp. by (i) the ability to grow on complex media and (ii) the inability to grow using C-1 compounds as the sole
carbon and energy source (Moore et al., 1984). Phylogenetic studies indicate that *Hyphomonas* spp. are distinct from *Hyphomicrobium* spp. (Stackebrandt et al., 1988; Nikitin et al., 1990).

By classical definition, *Hyphomicrobium* spp. are aerobic, methylotrophic organisms capable of anaerobic growth under denitrifying conditions with methanol as the sole carbon and energy source (Hirsch, 1989; Poindexter, 1992). This methylotrophic growth under denitrifying conditions has been employed as a selective enrichment technique for *Hyphomicrobium* spp. (Attwood and Harder, 1972). Some *Hyphomicrobium* spp. are capable of utilizing complex carbon sources such as glycerol, ethanol, succinate, and acetate (Hirsch, 1989). Some strains have been reported to contain aldehyde dehydrogenase capable of oxidizing benzaldehyde in whole-cell assays (Köhler and Schwartz, 1982). While the range of growth substrates suggests that *Hyphomicrobium* spp. are facultative methylotrophs, some strains have been described which are obligately methylotrophic (Izumi, 1982; Miyata, 1993). The range of C-1 compounds that *Hyphomicrobium* spp. are capable of utilizing as the sole carbon and energy source range from methanol and methylamine (Hirsch, 1989) to methyl chloride and dichloromethane (Hartmans et al., 1986; Stucki et al., 1981).

Methylotrophic organisms assimilate carbon via either the serine pathway or the ribulose monophosphate (RMP) pathway. *Hyphomicrobium* spp. assimilate carbon via the serine pathway. Isocitrate lyase (icl) is the enzyme responsible for catalyzing the cleavage of isocitrate to glyoxylate and succinate in the icl⁺ variant of the serine
pathway. However, *Hyphomicrobium* spp. growing with methanol or methylamine do
not have icl activity (Attwood and Harder, 1977; Anthony, 1982) and are, thus,
considered icl' variants of the serine pathway. The mechanism for the restoration of
glyoxylate to the serine icl' variant is currently unknown.

The enzyme 3-hexulose phosphate synthase is often used as an indication of
carbon assimilation via the RMP pathway (Anthony, 1982). This enzyme is
responsible for the incorporation of carbon as formaldehyde (Anthony, 1982). The
enzyme serine hydroxymethyltransferase is responsible for catalyzing the reaction
whereby carbon is assimilated as formaldehyde into the serine pathway. Serine
hydroxymethyltransferase is also present in organisms which do not possess the serine
pathway, being responsible for the generation of glycine from serine (Anthony, 1982;
Lidstrom, 1990). Hydroxypyruvate reductase, which catalyzes the reduction of
hydroxypyruvate to glycerate in conjunction with the oxidation of NADH to NAD⁺, is
often used as an indicator of serine pathway metabolism (Anthony, 1982; Krema and
Lidstrom, 1990). However, use of the hydroxypyruvate reductase assay can only be
used as an indication of serine pathway because low levels of hydroxypyruvate
reductase have been detected in some RMP pathway methylotrophs growing with
methanol (Bamforth and Quayle, 1977; Taylor et al., 1981). Recently, 16S ribosomal
RNA probes have been developed to distinguish between RMP pathway- and serine
pathway-utilizing bacteria (Brusseau et al., 1994).
For the present work, appendaged organisms were isolated from biofilms grown on glass slides immersed in municipal water wells with previous histories of Fe/Mn biofouling. One isolate, designated W1-1B, was characterized with respect to lifecycle, substrate utilization, fatty acid profile, and pathway of carbon assimilation.

**Materials and Methods**

Enrichment for *Hyphomicrobium* spp.

Water samples and biofilm samples retrieved from municipal water wells with histories of Fe/Mn biofouling problems were transferred to Medium 337 which consisted of (per L) KH$_2$PO$_4$, 1.36 g; Na$_2$HPO$_4$, 2.13 g; MgSO$_4$$\cdot$7H$_2$O, 0.3 g; (NH$_4$)$_2$SO$_4$, 0.5 g; CaCl$_2$$\cdot$2H$_2$O, 1.99 mg; FeSO$_4$$\cdot$7H$_2$O, 1.0 mg; MnSO$_4$$\cdot$H$_2$O, 0.35 mg; and Na$_2$MoO$_4$$\cdot$2H$_2$O, 0.5 mg, and contained 0.1% (v/v) of filter sterilized methanol (Hirsch, 1989). The enrichment cultures were incubated without shaking at room temperature (~22°C) until turbidity was observed. The cultures were examined by phase contrast microscopy for the presence of appendaged organisms. Those cultures containing appendaged organisms were reinoculated into fresh Medium 337 + 0.1% methanol. After 3 to 5 passes through Medium 337, enrichment cultures containing appendaged organisms were streaked onto solid Medium 337 (solidified with 1.5% (w/v) Bacto agar) and incubated at room temperature (~22°C). Colonies were examined under transmitted light. Those colonies which appeared brown and wrinkled under transmitted light were presumed to be *Hyphomicrobium* colonies. Wet mounts of
the colonies confirmed the presence of appendaged organisms. Those colonies were
inoculated into broth Medium 337 and then tested for purity on solid Medium 337.

From the enrichment cultures, 33 isolates were obtained. These isolates were
streaked onto Nutrient agar, Plate Count agar, and R2A agar to determine if the
organisms could grow on these media and to determine whether other non-
methylotrophic contaminants were present. The isolates were also streaked onto
Medium 337 with 5 g/L methylamine·HCl (non-volatile) as a carbon source. Medium
337 + 5 g/L methylamine + 2.5μg/L vitamin B12 (cyanocobalamin) were used for all
routine culturing of the isolates.

One isolate, designated as W1-1B, was chosen for further characterization. For
comparison, Hyphomicrobium zavarzinii (ATCC 27495), Hyphomicrobium vulgare
(ATCC 33404), and Hyphomicrobium DM2 (ATCC 43129) were obtained from the
American Type Culture Collection (Washington, D.C.).

The ATCC isolates and W1-1B were inoculated into Medium 337 + 0.5% (w/v)
methylamine·HCl + 0.2% (w/v) KNO₃ to test for the ability to use nitrate as a terminal
electron acceptor. The same medium was also used with methanol or dichloromethane
as the carbon source. After inoculation, the tubes containing medium and inverted
durham tubes were filled to the top with sterile medium and sealed with screw caps so
that there was no headspace. The tubes were incubated in the dark at room temperature
(~22°C) for up to 8 weeks and examined for turbidity and gas formation. After 8
weeks, the cultures were tested for residual nitrate by the addition of 6 drops of
diphenylamine solution (0.2 g diphenylamine in 100 mL of concentrated H$_2$SO$_4$) to one mL of culture (Tiedje, 1982). Appearance of a blue color indicated the presence of nitrate; no color development indicated the absence of nitrate.

Substrate Utilization Tests

Solidified Medium 337 without an added carbon source was used as a mineral salts base for substrate utilization tests. The following substrates were tested at 10 mM: glucose, succinate, acetate, pyruvate, formaldehyde, dichloromethane, methanol, methylvamine, dimethylamine, and trimethylamine. Dimethylsulfide was tested in the respective liquid medium. Control cultures contained the mineral salts base but no organic carbon source. All cultures were incubated at room temperature (~22°C) for 10 days.

Methylamine (5 g/L) was also tested as the sole carbon, energy, and nitrogen source.

Growth Curve

For a growth curve, isolate W1-1B was grown at ~22°C on a shaker in Medium 337 + 0.5% methylamine + 2.5 μg/L vitamin B12. A 1% initial inoculum of a 5 day old culture was used to inoculate fresh medium that would be sampled for the growth curve. Two separate replicates were run, and aliquots of the culture were periodically sampled over a 360 hr or 220 hr time period for optical density (660 nm). The
doubling time (g) was calculated during the logarithmic growth phase based on the
growth curve and specific growth rate constant (μ) was calculated based on the
equation $μ = \ln 2/g$.

Fatty Acid Profiling

Fatty acid profiles were obtained using a variation of the method of Miller and
Berger (1985) using a Hewlett-Packard HP 5898A Microbial Identification System
(HP-MIS). Cultures were streaked (using a three-phase streak pattern) onto plates of
337 medium containing 0.5% methylamine as the sole carbon source. The plates were
incubated at 30°C for 7 days. After incubation, the cells from the third quadrant were
harvested using a sterile inoculating loop. Enough cells were harvested to cover the
bottom of a 13 mm x 100 mm sterile screw cap tube. The cell samples were saponified
using 1.0 mL of saponification reagent (sodium hydroxide, 45 g; methanol, 150 mL,
deionized distilled water, 150 mL), mixing, and heating at 100°C for 30 minutes. The
samples were allowed to cool before adding 2.0 mL of methylating reagent (6.0 $N$ HCl,
325 mL; methanol, 275 mL). The fatty acid esters were methylated at 80°C for 10
minutes followed by rapid cooling ice. The samples were extracted into an organic
phase using 1.25 mL of extraction solvent (hexane, 200 mL, methyl-tert butyl ether,
200 mL), mixing for 10 minutes, and then decanting the bottom or aqueous phase. The
organic phase was washed using 3.0 mL of base wash (sodium hydroxide, 10.8 g;
deionized distilled water, 900 mL) and mixing for 5 minutes. The organic phase was
decanted into a gas chromatograph autosampler vial which was crimp sealed. The fatty acids were quantitated using the HP-MIS. *Klebsiella pneumoniae* and *Pseudomonas fluorescens* were used as reference standards.

Enzyme Assays

Cultures of *Hyphomicrobium W1-1B*, *H. zavarzinii*, *Methylophilus methylotrophus* AS1 (provided by R. S. Hanson, Gray Freshwater Inst., Navarre, MN 55392), and *Pseudomonas aeruginosa* PAO1 were harvested by centrifugation at 5000 x g for 10 minutes. The cells were resuspended in chilled (4°C) 0.5 M sodium potassium phosphate buffer (pH 7.5) containing 5 mM MgCl₂. For the hydroxypyruvate reductase assay, the cell suspensions were sonicated for 5 minutes. Cellular debris was removed by centrifugation at 12,000 x g for 20 minutes. The supernatant was considered the crude enzyme extract and was used undiluted in the enzyme assays. For the hexulose phosphate synthase assay, cells were disrupted using a French pressure cell at a pressure of approximately 5 MPa. Cellular debris was removed by centrifugation at 13,000 x g for 10 minutes. The supernatant was considered the crude enzyme extract, and 0.2 mL of the undiluted extract were used per assay.

Hydroxypyruvate reductase, responsible for the reduction of hydroxypyruvate to glycerate in conjunction with the oxidation of NADH to NAD⁺ in the serine pathway, was assayed for using the method of Krema and Lidstrom (1990). The enzyme assay
mixture consisted of 0.1 mL each of 38 mM (NH\(_4\))\(_2\)SO\(_4\), 4 mM NADH, 0.5 M sodium phosphate buffer (pH 7.5), and undiluted crude enzyme extract. The background absorbance at 340 nm was recorded before beginning the reaction by the addition of 0.1 mL of 2.0 mM Li-hydroxypyruvate. The absorbance at 340 nm of the assay mixture over a 15 minute period was recorded. A control containing no substrate (Li-hydroxypyruvate) and a control containing no enzyme extract were included in the assays.

Hexulose phosphate synthase, responsible for the incorporation of formaldehyde into the ribulose monophosphate pathway, was assayed using the method of Müller and Babel (1990). The assay mixture contained 1.2 mL of 100 mM sodium potassium phosphate buffer (pH 7.2), 0.1 mL of 60 mM MgCl\(_2\), 0.1 mL of 50 mM ribose-5-phosphate, 0.4 units of phosphoriboisomerase, and 0.1 mL of crude enzyme extract. This mixture was preincubated at 30°C for 30 minutes to generate an equilibrium between ribulose-5-phosphate and ribose-5-phosphate. The reaction was initiated by the addition of 0.1 mL of 50 mM formaldehyde. Aliquots of 50 \(\mu\)L were withdrawn periodically and quenched in 0.2 mL of trichloroacetic acid (50 g/L). The concentration of formaldehyde remaining was analyzed spectrophotometrically by the method of Nash (1953). Equal amount of sample was mixed with the colorimetric reagent (contains per L: 150 g ammonium acetate, 3 mL acetic acid, 2 mL acetylacetone) before incubating in a 50°C water bath for 20 minutes. The absorbance, read at 412 nm, correlated directly to the amount of formaldehyde in the sample.
Transmission Electron Microscopy (TEM)

For TEM, an aliquot of 10 µL of unwashed cell suspension of W1-1B was placed onto formvar, carbon-coated copper grids (300 mesh). The grids were placed in a petri dish and surrounded by several drops of water. This prevented the samples from drying as they were allowed to sit for 15 to 60 minutes to allow the cells to adhere to the grid. The grids were then rinsed several times by putting the grid into a drop of water. The non-coated side of the grid was blotted on a Kimwipe, and the grid was transferred to a drop of 1% (w/v) uranyl acetate for 30 seconds. The grid was then blotted dry and rinsed five times in drops of water. After allowing a few minutes for the grid to dry, the specimen was examined immediately using a Zeiss 10 transmission electron microscope. The above protocol was obtained from Y. Brun (Dept. of Biology, Indiana Univ., Bloomington, IN 47405).

For thin sectioning, the method based on Cagle et al. (1972) was used. The cells were washed with 0.1 M sodium cacodylate buffer (pH 7.4) three times. Cells were pelleted, washed twice in 0.1M cacodylate buffer, and washed once in 0.15% ruthenium red + 0.1 M cacodylate buffer (RRC buffer). The cells were resuspended in 4.0% glutaraldehyde and 0.15% ruthenium red. After one hour of fixation, the cells were washed 2 times in RRC buffer. Immediately after washing, the pellet was mixed with 2 drops of 1.5% Bacto agar (Difco) and allowed to solidify. Blocks of 0.5 mm³ were cut from the agar plug. The small blocks were dehydrated in an ethanol:water
series (30%, 50%, 70%, 90%, and 95% ethanol) before placing into 100% ethanol. The residual ethanol was removed by immersing the blocks in propylene oxide for 10 minutes. The sample blocks were then embedded in Epon 812 before cutting thin sections. Thin sections were stained with lead citrate and uranyl acetate and examined under a Zeiss 10 transmission electron microscope.

Results

A total of 33 appendaged, methylotrophic isolates were obtained from the enrichment cultures. The colonies selected from streak plates were those that appeared brown and wrinkled under transmitted light (Figure 14a.) Under reflected light, these colonies appeared creamy white in color (Figure 14b). Under transmitted light, the presumptive *Hyphomicrobium* colonies could be distinguished from other organisms because the other organisms appeared white or clear in color while the presumptive *Hyphomicrobium* colonies appeared dark brown (Figure 15).

Of the 33 appendaged, methylotrophic isolates obtained, one isolate, designated W1-1B was chosen for further characterization. The major reason for the selection of this isolate was that the nascent swarmer cells were highly motile, as observed by phase contrast microscopy. For the substrate utilization tests, W1-1B was compared to the ATCC cultures of *Hyphomicrobium* DM2, *H. zavarzinii*, and *H. vulgare*. Of the substrates tested, W1-1B grew with methanol, methylamine, dimethylamine, and trimethylamine. The results for all of the substrate utilization tests are presented in Table 5. All four organisms were capable of utilizing methylamine as the sole carbon,
Figure 14. Colonies of W1-1B growing on Medium 337 + 0.5% (w/v) methylamine plates: a) under transmitted light and b) under reflected light.
Figure 15. Colonies of presumptive *Hyphomicrobium* colonies (brown or dark) and non-*Hyphomicrobium* bacterial contaminants (white or light) growing on Medium 337 + 0.5% (w/v) methylamine plates.

<table>
<thead>
<tr>
<th>Test Substrate</th>
<th>W1-1B</th>
<th><em>H. vulgare</em></th>
<th><em>H. zavarzinii</em></th>
<th><em>Hyphomicrobium</em> DM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No carbon</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Succinate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Pyruvate</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methylamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* - = no growth, + = growth
energy, and nitrogen source.

The cultures were also tested under denitrifying conditions using either methanol or methylamine as the carbon source. Increase in turbidity and the production of gas trapped in durham tubes suggested that Hyphomicrobium DM2, H. zavarzinii, and H. vulgare were capable of anaerobic growth. Microscopic examination of these cultures revealed the presence of appendaged cells. The W1-1B culture did not have the presence of turbidity and gas bubbles were not formed. After the addition of diphenylamine to samples from all the cultures, no color appeared in samples from Hyphomicrobium DM2, H. zavarzinii, and H. vulgare, indicating that no residual nitrate was present whereas addition of diphenylamine to W1-1B samples produced a dark blue color. None of the organisms was able to grow under denitrifying conditions using dichloromethane as the carbon source.

Construction of a growth curve for W1-1B while growing on Medium 337 supplemented with 5 g/L methylamine and 2.5 μg/L vitamin B12 provided data to calculate the doubling time and the specific growth rate constant of the organism (Figure 16). The average doubling time (average of the two curves) or time necessary for the optical density of the culture to double during logarithmic growth was 22.2 hrs. Under the described conditions, μ was 0.031 hr⁻¹ for W1-1B.

Analysis of fatty acid profiles by HP-MIS was not able to match Hyphomicrobium DM2, H. zavarzinii, H. vulgare, or W1-1B to any profiles in the data base. Instead, a new entry for Hyphomicrobium spp. was created using the combined profiles from Hyphomicrobium DM2, H. zavarzinii, and H. vulgare. The individual
Figure 16. Growth curve of W1-1B growing on Medium 337 + 0.5% (w/v) methylamine + 2.5 μg/L vitamin B12 on a shaker table at room temperature (~22°C).
profiles of *Hyphomicrobium* DM2, *H. zavarzinii*, *H. vulgare*, and W1-1B were then compared against the *Hyphomicrobium* spp. entry. The match number for *H. zavarzinii* was 0.677, for *H. vulgare* was 0.646, for *Hyphomicrobium* DM2 was 0.321, and for W1-1B was 0.783. A match number of 0.50 or higher is an acceptable match. In addition, the majority of the fatty acid composition of the organisms was 18:1 *cis* fatty acids, with the composition of *H. zavarzinii* 78.8%, *H. vulgare* 80.19%, *Hyphomicrobium* DM2 76.31%, and W1-1B 74.47%.

Hydroxypyruvate reductase activity over time for W1-1B, *H. zavarzinii*, *P. aeruginosa* PAO1, and *M. methylotrophus* AS1 is presented in Figure 17. Both W1-1B and *H. zavarzinii* exhibited hydroxypyruvate reductase activity over the 15 minute assay but *P. aeruginosa* PAO1 and *M. methylotrophus* AS1 did not. Controls containing no substrate and no enzyme extract did not show a significant decrease in absorbance in the assay. Results from the hexulose phosphate synthase assay were inconclusive. Hexulose phosphate synthase activity was not detected in *M. methylotrophus* AS1.

TEM of whole cells was used to examine the lifecycle of *Hyphomicrobium*. Figure 18a is representative of a non-appendaged nascent cell. Typically, cells at this stage of the lifecycle are motile by a single sub-polar flagellum (Harder and Attwood, 1978). The cell in Figure 18a does not contain any flagella although phase contrast microscopy of the culture used before fixation indicated that motile cells were present. Cell-free flagella were observed on the grids. The absence of any cell-attached flagella in Figure 18 may be due to the fact that the cell had already reached the point in its
Figure 17. Plot of hydroxypyruvate reductase activity measured as the oxidation of NADH to NAD$^+$ as hydroxypyruvate is reduced to glycerate by hydroxypyruvate reductase.
Figure 18. Electron micrographs of non-appendaged, nascent cells of W1-1B. (A) Bar = 100 nm, (B) = 50 nm.
lifecycle where the flagellum was shed or that the shear stresses of fixation for electron microscopic examination tore any flagella present from the cells. Figure 18b represents a cell that is beginning to form an appendage. Elongations are present from both ends of the cell body (Figure 18b). Figure 19 is a representation of a cell beginning formation of an appendage. A cell with an elongated appendage is presented in Figure 20. An appendaged cell is presented in Figure 21. There is a slight swelling at the distal end of the appendage where daughter cells form. Figure 22 is a representation of a cell with two appendages. It was not uncommon to see an occasional cell with more than one appendage.

Photomicrographs of thin sections of cells at various points throughout the lifecycle were obtained. Figures 23 and 24 represent non-appendaged cells with areas of elongation. Figure 25 represents an appendaged cell. An appendaged cell with swelling at the distal end of the appendage is presented in Figure 26. The structure of a Gram negative cell wall is evident in the cross section of the cell in Figure 27.

Discussion

Because of the differential lifecycle, ability to utilize methylotrophic compounds, C18:1 fatty acid composition, and hydroxypyruvate reductase activity W1-1B was classified as a *Hyphomicrobium* sp. The inability of W1-1B to grow under anaerobic conditions using nitrate as a terminal electron acceptor is contrary to previous definitions of *Hyphomicrobium* spp. Still, based on the other results, W1-1B fits best into the genus of *Hyphomicrobium*. Therefore, by relying solely on denitrifying
Figure 19. Whole cell of W1-1B with elongation of the appendage. Bar = 50 nm.
Figure 20. An appendaged cell of W1-1B. Bar = 50 nm.
Figure 21. A mature cell of W1-1B with an elongated appendage. Bar = 50 nm.
Figure 22. Cells of W1-1B that have more than one appendage. Bar = 50 nm.
Figure 23. Thin section representative of a non-appendaged, nascent W1-1B cell. Bar = 100 nm.
Figure 24. Thin section representative of a cell beginning elongation of the appendage of W1-1B. Bar = 100 nm.
Figure 25. Thin section representative of an appendaged cell of W1-1B. Bar = 100 nm.
Figure 26. Thin section representative of a mature W1-1B cell with a slight swelling at the distal end of the appendage. Bar = 100 nm.
Figure 27. Thin section of a W1-1B cell. Note the Gram-negative cell wall structure. Bar = 100 nm.
cultures as enrichments for *Hyphomicrobium* spp. (Attwood and Harder, 1972), some non-denitrifying strains of *Hyphomicrobium* could be overlooked.

Isolate W1-1B was only able to grow using C-1 compounds as the sole carbon and energy source. Hydroxypyruvate reductase activity was detected in W1-1B and *H. zavarzinii* suggesting the presence of the serine pathway of C-1 metabolism. The isolate W1-1B, *H. vulgare*, and *H. zavarzinii* were incapable of utilizing dichloromethane. Only *Hyphomicrobium* DM2 was capable of growth using dichloromethane as the sole carbon and energy source, in keeping with the dichloromethane dehalogenase which has been characterized in this organism (Stucki et al., 1981; Kohler-Staub and Leisinger, 1985; Kohler-Staub et al., 1986).

All of the strains were able to use methanol, methylamine, dimethylamine, and trimethylamine. Among the other substrates tested, only *Hyphomicrobium* DM2 and *H. zavarzinii* were able to utilize acetate. According to the most recent classification scheme (Hirsch, 1989), *H. vulgare* should be able to utilize succinate and acetate but this is not confirmed in the present work. The information in Bergey's Manual of Systematic Bacteriology (Hirsch, 1989) is based on data which were collected nearly 100 years ago with a *H. vulgare* type strain which is no longer available (Hirsch, 1989). The classification of the genus *Hyphomicrobium* is in need of refinement.

The ability of *H. vulgare* and *H. zavarzinii* to grow on methanol and methylamine under denitrifying conditions is as expected. However, the observed growth of *Hyphomicrobium* DM2 with methanol under denitrifying conditions is
contrary to previous results (Stucki et al., 1981). The diphenylamine assay used for the qualitative detection of NO$_3$-N and NO$_2$-N is sensitive to a detection limit of approximately 200 ppb (J. J. Crawford, Dept. of Agronomy, Univ. of Illinois, Urbana, IL 61801, personal communication).

The doubling time of 22.2 hr for W1-1B when grown on methylamine was longer than those reported for other *Hyphomicrobium* strains (Matzen and Hirsch, 1982a). In the previous studies, however, Matzen and Hirsch (1982a) did not specify whether methanol or methylamine was used as the substrate for determination of the $g$ values. The specific growth rate constant ($\mu$) for *Hyphomicrobium* spp. growing on methanol is $<0.1$ hr$^{-1}$, and only one isolate was reported to have a $\mu$ value of about 0.2 hr$^{-1}$ (Takada, 1975). The $\mu$ value for methylamine-grown W1-1B was $<0.1$ hr$^{-1}$. For *Hyphomicrobium* DM2, the $\mu$ value was 0.1 hr$^{-1}$ with methanol and 0.07 hr$^{-1}$ with dichloromethane as the substrate (Stucki et al., 1981).

The major fraction of cellular fatty acids in W1-1B is C18:1, in agreement with published reports of *Hyphomicrobium* spp. fatty acid composition (Urakami and Komagata, 1987a; Urakami and Komagata, 1987b; Guckert et al., 1991). Urakami and Komagata (1979) assigned methylotrophic organisms with primarily C16:0 saturated and C16:1 unsaturated fatty acids to Type A and those with primarily C18:1 fatty acids as Type B. Guckert et al. (1991) concluded that the phenotypic relationships based on the phospholipid, ester-linked fatty acid profiles correlated well with the respective analysis based on 16S rRNA sequence. The fatty acid composition of
Hyphomicrobium spp. is closely comparable to that of Hyphomonas spp., a non-methylo trophic, appendaged organism and of Rhodomicrobium vannielii, a photosynthetic, appendaged organism (Urakami and Komagata, 1987b). These three genera can be differentiated on the basis of the composition of the major cellular fatty acids and the major hydroxy acids (Urakami and Komagata, 1987b).

The lifecycle of W1-1B was comparable to those previously described for Hyphomicrobium spp. (Harder and Attwood, 1978; Brock and Madigan, 1991). All morphological cell types were observed during the lifecycle except for a nascent, swarmer cell with an attached flagellum. Thin sections indicated a Gram-negative cell wall structure, and this structure was seen in all micrographs of the cell bodies and the appendages. The appendage was an elongation of the cell itself and was surrounded by a continuous cell wall, as previously reported (Moore and Hirsch, 1973). Electron microscopic examination did not indicate the presence of extracellular polysaccharide material in ruthenium red treated samples.

A phylogenetic analysis of W1-1B is presently underway. Previous studies on DNA-DNA homologies of several Hyphomicrobium strains have suggested considerable diversity in this genus as well as in the genera Hyphomonas and Pedomicrobium (Gebers et al., 1985; Gebers et al., 1986). Stackebrandt et al. (1988) published phylogenetic analysis of 16S rRNA for several Hyphomicrobium strains which place Hyphomicrobium spp. in the alpha subdivision of the purple bacteria. Variation in 16S rRNA relationships also indicate a large degree of diversity in the
genus *Hyphomicrobium* (Stackebrandt et al., 1988). Based on published sequences, *Hyphomicrobium* spp. have few conserved 16S rRNA sequences with other methylotrophic bacteria (Brusseau et al., 1994; R. S. Hanson, Gray Freshwater Inst., Univ. of Minnesota, Navarre, MN 55392, personal communication).

Based on these data and the available reference information, the isolate W1-1B was classified as a *Hyphomicrobium* spp. Addition of the 16S rRNA data will be useful in further confirming classification of this organism as well as add to the existing data involving the phylogenetic classification of *Hyphomicrobium* spp.
CHAPTER VI

CHEMOTAXIS, MOTILITY, AND FLAGELLAR STRUCTURE OF
HYPHOMICROBIUM W1-1B

Abstract

Motile swarmer cells of Hyphomicrobium W1-1B displayed a positive, chemotactic response toward methylamine, dimethylamine, and trimethylamine. The response was not inducible and appeared to be mediated by a single chemoreceptor. Methanol and arginine did not elicit significant chemotactic responses. Video microscopy of anchored swarmer cells indicated that the flagella of Hyphomicrobium W1-1B generally rotated counterclockwise but were also capable of clockwise rotation and temporarily stopping all rotation. The flagella of Hyphomicrobium W1-1B were composed of two proteins of 39,000 $M_r$ and 41,000 $M_r$, neither of which appeared to be a glycoprotein as determined by Schiff's staining or by enzyme immunoassay. Protein fingerprints visualized from protease-digested samples of each protein using silver-stained polyacrylamide gels and Western blots to determine the relatedness of the proteins indicated that the proteins were similar but not identical.
Introduction

During the lifecycle, nascent daughter cells of *Hyphomicrobium* spp., referred to as swarmer cells, are motile by a single subpolar flagellum (Moore, 1981). The cells mature after shedding of the flagella followed by the formation of an appendage, and the mature cells are capable of producing nascent, swarmer cells from the distal tip of the appendage (Poindexter, 1992).

Studies of the lifecycle of *Hyphomicrobium* spp. have often focused on the nascent swarmer cells which have been exploited in the formation of synchronous cultures (Moore and Hirsch, 1973; Matzen and Hirsch, 1982b). The swarmer cells of *Hyphomicrobium* strain B522 remain motile for 5-10 hours before shedding their flagella and initiating appendage formation (Moore and Hirsch, 1973). Swarmer cells of *Hyphomicrobium* strain ZV580 may reach velocities of 25-30 μm/s when in Medium 337 at 30°C (Moore, 1981). Swarmer cells of *Hyphomicrobium* strain ZV580 have remained motile for up to 24 at 4°C (Moore and Marshall, 1981).

The structure of the flagellum has not been previously characterized. Only anecdotal evidence of the chemotaxis of *Hyphomicrobium* spp. to an air/medium interface has been described (Moore and Marshall, 1981). *Hyphomicrobium* spp. have been observed previously in biofilm samples (Tuhela et al., 1993), and their flagella may participate in the attachment of the cells to surfaces (Moore and Marshall, 1981). Swarmer cells of *Hyphomicrobium* strain ZV580 attached reversibly to glass surfaces by means of their flagella (Moore and Marshall, 1981). It has been suggested that
Hyphomicrobium strain ZV580 cells attach to surfaces with the aid of an adhesin which has properties similar to those of a glycoprotein or a peptidopolysaccharide (Moore and Marshall, 1981). Moore and Marshall (1981) suggested that the flagella may provide the means of transport of this adhesin from the cell to the surface, thus facilitating the attachment of Hyphomicrobium cells to surfaces.

Of the appendaged organisms, only Caulobacter spp. have been characterized with respect to chemotactic response and structure of the flagella. The filament of Caulobacter flagella are composed of at least three different flagellin proteins (Brun et al., 1994). Production of these flagellin proteins is temporally regulated, and the proteins are spatially distributed in the predivisional cell from the appendaged cell to the swarmer cell. Comparison of the chemotactic response and flagellar structure of other appendaged organisms to that of Caulobacter spp. may provide insight to spatial and temporal regulation in swarmer cells of appendaged organisms.

The present work was aimed at studying a part of the cell cycle of Hyphomicrobium W1-1B, particularly the swarmer cell. To date, this is the first report to characterize the chemotaxis and flagellar structure of a Hyphomicrobium sp.

Materials and Methods
Harvesting cells for taxis experiments

Swarmer cells for chemotaxis experiments were grown in 500 mL of Medium 337 and supplied 0.5% (w/v) either methylamine, dimethylamine, or trimethylamine as
the sole carbon and energy source. Cultures were incubated at ~22°C with no shaking for 72 hours. The cells were harvested by centrifugation at 3000 x g for 10 minutes and washed twice with 337 buffer (per L: KH₂PO₄, 1.36 g; Na₂HPO₄, 2.13 g; MgSO₄·7H₂O, 0.3 g; CaCl₂·2H₂O, 1.99 g; FeSO₄·7H₂O, 1.0 mg; MnSO₄·H₂O, 0.35 mg; and Na₂MoO₄·2H₂O, 0.5 mg). Washed pellets were resuspended in 15 mL of 337 buffer.

Chemotaxis Assays

Chemotactic responses were assayed using a modified capillary assay technique (Adler, 1973). The chemotaxis chambers were constructed of 55 mm x 55 mm Lucite plastic blocks and contained four sets of wells, each 7 mm in diameter and 5 mm in depth. Each set of wells was joined by a narrow channel, 23 mm in length. The assay was conducted by filling the wells and channel with washed, motile cells in 337 buffer and placing a 1 µL capillary tube filled with a specific concentration of an attractant or the control buffer in the channel. The attractants tested were methylamine, dimethylamine, and trimethylamine. Each assay was done in replicates of three or four. The assembled assay was incubated at ~22°C for 60 minutes. After incubation, the capillary tubes were removed from the assay chambers, rinsed with sterile water, and the contents of the capillary tubes were expelled into buffer which was then plated onto Medium 337 + 0.5% methylamine agar plates using a model DU spiral plater (Spiral Systems, Inc., Cincinnati, OH). The plates were incubated at ~22°C for 7 days.
before counting. Assays for each attractant were done in duplicate.

Competition chemotaxis assays were used to determine whether more than one receptor was involved in the chemotactic response toward methylamine, dimethylamine, and trimethylamine. Swarmer cells grown with dimethylamine as the sole carbon and energy source were washed and resuspended in 1 mM of methylamine, dimethylamine, or trimethylamine. In each case, 1 mM solutions of methylamine, dimethylamine, and trimethylamine were placed in capillary tubes and seated in the chemotaxis chambers. All assays were done in duplicate with either three or four replicates per treatment. The remainder of the competition assay was then identical to all other chemotaxis assays previously described here.

Tethering Technique

Tunnel slides were prepared using microscope slides and cover slips. Vacuum grease was used to secure two cover slips to a microscope slide such that there was a gap of about 0.5 cm between the two. A third cover slip was cleaned by immersing in KOH-saturated 95% ethanol, then immersing in nanopure water, and finally by rinsing in a stream of nanopure water. The ultra-clean cover slip was attached with vacuum grease to the slide so that the gap between the previously attached cover slips was bridged, forming a tunnel slide.

When monoclonal antibodies were used for anchoring the cells, various concentrations of antibody were placed inside the tunnel and allowed to attach to the
glass by incubating on ice for 30 minutes. Buffer was then used to remove any unattached antibody. The motile cells suspension was then wicked under the tunnel. After 10 to 15 minutes, the unattached cells were removed using buffer. The slides were observed under phase contrast microscopy. When antibodies were not used to anchor the cells, the cell suspension was placed under the tunnel and allowed to incubate at room temperature for 10 to 15 minutes to allow attachment of the cells. Various concentrations of methylamine (from 100 mM to 1 mM) were wicked under the tunnel to observe the direction of rotation of the cells.

After anchoring of the cells, the slides were observed using a Zeiss inverted microscope. Selected fields were video taped to characterize the direction of flagellar rotation.

Flagella Preparation

*Hyphomicrobium* W1-1B was grown in 10 L fermentor batches containing 337 medium with either 0.5% (w/v) methylamine or 0.5% (v/v) filter-sterilized methanol as the sole carbon and energy source. A 1% (v/v) inoculum of *Hyphomicrobium* W1-1B was used. The fermentors were incubated at ~22°C with aeration but no stirring for 72 hours. Cells were harvested by first concentrating with a Pellicon filter and then pelleting the cells by centrifugation at 3000 x g and 4°C for 15 minutes. The cells were resuspended in no more than 30 mL of 337 buffer.
Flagella were sheared from the cells using a blender at the maximum speed for 15 seconds. After shearing, the cells were observed microscopically to ascertain that cells were no longer motile. Cells were separated from the freed flagella by centrifugation at 10,000 x g at 4°C for 12 minutes. The resultant supernatant was again spun at 15,000 x g at 4°C for 12 minutes to remove remaining cells debris from the flagella. Flagella were then pelleted by ultracentrifugation at 100,000 x g for 2 hours at 4°C with a decel of 0. The pellet was resuspended in up to 100 μL of HEC buffer and kept at 4°C.

Protein Assay

The protein content of flagella preparations was determined using the Micro BCA Protein Assay Reagent kit (Pierce Chemical Company, Rockford, IL). A standard curve was prepared from bovine serum albumin (BSA) in HEC buffer (containing 10 mM HEPES at pH 7.0, 10 μM EDTA, and 0.2 mM CaCl₂) at concentrations of 0, 1.0, 2.5, 10 and 20 μg protein/mL. For each flagella preparation sample, a 10 μL aliquot was combined with 990 μL of HEC buffer. The assay was performed according to manufacturer's instructions and absorbance values were read at 562 nm.

Sucrose Density Gradient

Flagella preparations were purified using sucrose density gradient centrifugation. Ultracentrifuge tubes were rinsed with distilled demineralized water
before layering in 50%, 40%, and 30% (w/w) sucrose solutions at 4°C. Approximately 4 mL of each sucrose solution was layered into the tubes. The flagella preparation sample was layered onto the top of the sucrose layers. If the volume of the flagella preparation was less than 500 μL, the total volume of the sample was brought up to 500 μL using chilled HEC buffer. The sample was separated by ultracentrifugation at 100,000 x g at 4°C for 18 hours with a decel of 0.

After centrifugation, the contents of the sucrose density gradient were separated by fractionation. The ultracentrifuge tube was placed into a fraction collector, and the contents of the tube were displaced by flushing the tube with Fluorinert (DuPont). The sample passed through a UV detector and the results were recorded on a chart recorder set at a current of 300, sensitivity of 0.02, and chart speed of 60 cm/hour. Fractions of 12 drops were collected in eppendorf tubes. There was about a 1 mL lag time from the recording of a sample on the chart recorder to the actual collection of the sample.

To measure the index of refraction of the fractions, a refractometer (Bausch & Lomb, Rochester, NY) was used. Samples (130 μL) were placed onto the sample crystal and the index of refraction was recorded.

Negative Stains of Flagella

A 1:10 dilution of the flagella preparation was prepared. From this dilution, 10 μL was placed onto a formvar, carbon-coated grid and allowed to air dry. A drop of 1% uranyl acetate was then placed onto the grid and allowed to sit for 30 to 60 seconds.
The grid was washed several times in demineralized, distilled water, blotted dry, and then air dried. Samples were observed using a Zeiss 10 transmission electron microscope.

SDS-PAGE

Proteins were separated using SDS-PAGE. Resolving gels were cast with a final concentration of 12% acrylamide and stacking gels were cast with a final concentration of 5% acrylamide using a 30:0.8 acrylamide to bis acrylamide solution (Amresco, Solon, OH), unless otherwise noted. Resolving gels were diluted in a 1.5 M Tris buffer, pH 8.8, containing 0.1% (w/v) SDS while stacking gels were diluted in a 0.5 M Tris buffer, pH 6.8, containing 0.1% (w/v) SDS. The addition of ammonium persulfate and tetraethylethylene diamine (TEMED) initiated the polymerization of the acrylamide. Details are presented in the Appendix. Protein samples were denatured by mixing 1:1 with the 2X cracking buffer and heating at 95°C for 5 minutes. The samples were loaded onto the gel which was placed into a Mini-Protean II cell (Bio-Rad Laboratories) containing running buffer. Separation by electrophoresis was performed at 200 V until the dye front reached the bottom of the gel, usually in 45-60 minutes. Details for the buffer compositions and casting of gels are presented in the Appendix.

The High Molecular Weight Range standards from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD) were run alongside the sample proteins for
comparison. The standards consisted of myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa). The Mid-Range Protein Molecular Weight Markers (Promega Corporation) were used for protein fingerprinting and consisted of phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (42.7 kDa) aldolase (40 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Kaleidoscope Prestained Standards (Bio-Rad Laboratories) were used for Western blotting and contained myosin (207 kDa), β-galactosidase (139 kDa), bovine serum albumin (84 kDa), carbonic anhydrase (41.7 kDa), soybean trypsin inhibitor (32 kDa), lysozyme (17.9 kDa), and aprotinin (8.6 kDa).

Staining of Polyacrylamide Gels

Polyacrylamide gels were stained using Coomassie stain which consisted of 0.2% (w/v) Coomassie Blue R, 45% (v/v) methanol, and 10% (v/v) acetic acid in distilled deionized water. After staining for between 60 to 120 minutes, the gels were rinsed briefly with distilled water and then destained with a solution consisting of 5% (v/v) methanol and 7.5% (v/v) acetic acid in distilled deionized water. Several changes of destain solution were used, and destaining was continued overnight or until the desired background was obtained.
For protein fingerprinting, secondary polyacrylamide gels containing the digested protein peptides were silver stained as previously described (Deakin, 1994). Initially, the gel was soaked in a solution of 5% (v/v) formaldehyde and 40% (v/v) ethanol for 30 minutes. The gel was then soaked in distilled demineralized water for 30 minutes before drying in 50% (v/v) methanol for 2 to 3 hours or overnight. Afterwards, the gel was rehydrated in distilled demineralized water containing a dash of dithiothreitol for 30 minutes, followed by soaking in a 0.1% (w/v) AgNO₃ solution for 30 minutes. After washing twice, briefly, with distilled demineralized water, a developer (consisting of 10 g sodium carbonate and 300 μL formaldehyde per L water) was added with gentle shaking until the desired level of color intensity was obtained. The developer was changed as it formed a brownish color. The color development was stopped with the addition of the stop solution (containing 10 g citric acid in 20 mL of water). After the fizzing ceased, the gel was transferred to distilled demineralized water for storage until drying.

Gels were dried on a 25 cm x 28 cm gel drying frame. Before drying, the gels were soaked in 3% (v/v) glycerol for 3 to 4 hours. They were then placed between two sheets of cellulose which were prewetted with distilled demineralized water. The frame was clamped together, and the gels were allowed to dry horizontally overnight.
Glycosylation Detection

The two proteins associated with the flagella of *Hyphomicrobium* W1-1B were tested for the presence of glycosylation using the First Choice Glycan Detection kit (Boehringer Mannheim Corporation, Indianapolis, IN) which is an enzyme immunoassay for the detection of glycoconjugates. Samples and controls were separated by SDS-PAGE. Transferrin was used as a positive control and creatinase was used as a negative control. For both controls and the test sample, 1.0 µg of protein was added per well. The sample and controls were run in duplicate, and after electrophoresis, the gel was cut in half so that one half was stained with Coomassie stain and the other was transferred onto nitrocellulose by Western blotting. The protein on the nitrocellulose was treated and probed according to kit directions in order to visualize antibody-glycoprotein complexes.

To further test the two flagella-associated proteins from *Hyphomicrobium* W1-1B for glycosylation, Schiff's staining procedure was used (Gerard, 1990). Transferrin was used as a positive control and creatinase was used as a negative control. The proteins, 5 µg each, were separated by SDS-PAGE. The gel was incubated in 2 N HCl containing 5% (w/v) phosphotungstic acid for 90 minutes, then washed twice for 60 minutes each in a 7% methanol/14% acetic acid solution. After washing, the gel was incubated in a 1% periodic acid/7% trichloroacetic acid solution for 60 minutes, and excess periodic acid was removed by incubating the gel an addition 60 minutes in 0.1 N HCl containing 0.5% sodium metabisulfite. The gel was stained using Schiff's reagent
(Sigma Chemical Company, St. Louis, MO) at 4°C in the dark overnight.

Protein Fingerprints

The relatedness of the two proteins associated with *Hyphomicrobium* W1-1B flagella was compared using the Protein Fingerprinting System kit (Promega Corporation). Flagellar proteins were separated in a primary gel using SDS-PAGE. Primary gels were standard Laemmli gels and were cast with a 12% acrylamide resolving gel and a 2 cm 4.5% stacking gel. The gel was 7 cm x 9 cm and was 0.75 mm thick. Approximately 3 μg of protein was mixed with the 2x cracking buffer and heated at 95°C for 5 minutes. The control protein, phosphorylase B, was supplied in the Protein Fingerprinting System kit and was prepared according to manufacturer’s instructions. After loading, the separation by electrophoresis was conducted using the Bio-Rad Miniprotein II apparatus at a voltage of 200 V until the dye front reached the bottom of the gel. At this point, the gel was stained for 10 minutes with a modified Coomassie stain (containing 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid, and 0.25% (w/v) Coomassie Brilliant Blue R250) and destained with 7% (v/v) glacial acetic acid for 30 minutes. After destaining, the target protein bands were cut from the gel with a razor blade. The gel slices were placed in petri dishes containing approximately 15 mL of running buffer and equilibrated for 30 minutes before loading onto the secondary gel. The secondary gel had a 15% acrylamide resolving gel and a 2.5 cm resolving gel of 4.5% acrylamide. The gel was 7 cm x 9 cm and was 1.0 mm thick.
The gel slices were loaded into individual wells using forceps. Each gel slice was overlaid with 5 μL of the provided gel slice overlay solution and 5 μL of the appropriately diluted protease. The supplied lyophilized proteases were rehydrated according to manufacturer's directions and diluted with the supplied 5x protease diluent solution and nanopure water such that the 5 μL overlay represented a protease/protein ratio (w/w) of 2% for alkaline protease, 5% for endoproteinase Glu-C, and 10% for endoproteinase Lys-C. Electrophoresis was conducted at 100 V for 20 minutes, stopped for 15 minutes to allow for digestion, and then continued at 100 V until the dye fronts reached the stacking gel/resolving gel interface. The voltage was discontinued for an additional 15 minutes before resuming electrophoresis at 200 V until the dye front reached the bottom of the gel. The gel was cut in half with a razor blade such that one half was stained with the silver stain method and an identical half was transferred to a nitrocellulose membrane by Western blotting using a BioRad transblot apparatus. The blotted proteins were probed with a monoclonal antibody which was specific to *Rhizobium meliloti* RMB7201 flagella and was previously shown to cross-react with *Hyphomicrobium* W1-1B flagella.

Preparation of Monoclonal Antibodies

Sucrose banded flagella preparations from *Rhizobium meliloti* RMB7201 were used as the antigen. The monoclonal antibodies were prepared at the Monoclonal Antibody Facility at The Ohio State University (Columbus, OH 43210). A total of four
BALB/c mice immunized with Freund’s complete adjuvant were injected with 15 μg each of the antigen in three intervals. The spleens were harvested and fusions were made using a P3x63 AG853 myeloma cell line. Clones were tested by ELISA, and the supernatant of the clones positive against the antigen were tested by Western blots. A second cloning was done and 500 mL of the hybridoma medium was purified for antibodies. Saturated ammonium sulfate solution (4.1 M, pH 7.0) was mixed slowly with 250 mL of the fusion tissue culture supernatants to achieve a 50% saturation of the supernatant in order to precipitate the antibodies. The entire mixture was stored overnight at 4°C, after which the mixture was separated by centrifugation at 3000 x g to 30 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 40 mL of phosphate buffered saline. This mixture was dialyzed against three changes, 2 L each, of phosphate buffered saline at 4°C over 48 hours. The purified antibody solution was stored at -70°C.

The specificity of the monoclonal antibody was screened by testing for cross-reactivity with denatured flagella from *R. meliloti, Hyphomicrobium W1-1B, Agrobacterium tumefaciens, Bradyrhizobium japonicum, Pseudomonas aeruginosa PAO1, and M91-3*, an unclassified, atrazine-degrading, Gram-negative soil bacterium (Radosevich et al., 1995). The proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters by the method previously mentioned. The Western blot was probed with the monoclonal antibody by the method described in the following paragraph.
Western Blots

Polyacrylamide gels were blotted onto 0.45 μm pore size nitrocellulose membranes (Schleicher & Schuell, Keene, NH) using a Bio-Rad Mini Trans-Blot Cell (Bio-Rad Laboratories). The transfer buffer consisted of 25 mM Tris base, 190 mM glycine, and 20% (w/v) methanol which was degassed 30 minutes before adding 0.1% (w/v) sodium dodecyl sulfate. Blots were run at 250 mA at 4°C for 2 hours. If not immediately visualized using immunodetection, the nitrocellulose membranes were air dried for 1-2 minutes, wrapped in plastic wrap and aluminum foil, and stored at 4°C.

Proteins blotted onto nitrocellulose membranes were exposed to monoclonal antibodies specific to *Rhizobium meliloti* 7201 and then visualized using the ProtoBlot II AP System with Stabilized Substrate, Mouse (Promega Corporation, Madison, WI). Buffers used in the immunodetection were Tris buffered saline (TBS) which consisted of 20 mM Tris·HCl (pH 7.5), and 150 mM NaCl; Tris buffered saline with Tween (TBST) which consisted of 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 0.05% (v/v) Tween 20 (Promega Corporation); and blocking solution which consisted of 1.0% (w/v) blot qualified bovine serum albumin (Promega Corporation) in TBST. The standard protocol for the immunodetection of the Western blot began with blocking nonspecific binding sites on the membrane with blocking solution for 30 minutes. Following the blocking step, the membrane-bound proteins were exposed to the primary antibody (diluted 1/100 in TBST) for 60 minutes with gentle shaking.
Afterwards, the membrane was washed with three times, 5 minutes each, with TBST to remove unbound primary antibody. The membrane-bound protein was next exposed to the anti-IgG AP conjugate, or secondary antibody (diluted 1/3000 in TBST), for 30 minutes with gentle shaking. Unbound secondary antibody was removed with three washes, five minutes each, with TBST. Any residual Tween 20 was removed with two washes, one minute each, with TBS. The cross-reacted of proteins were visualized by adding Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega Corporation) and incubating with gentle shaking for 15 minutes to 24 hours, or until color development was complete. The color reaction was stopped by washing twice with double distilled deionized water. Membranes were stored in plastic bags after drying on blotting paper.

Results

When grown on methylamine, dimethylamine, or trimethylamine, *Hyphomicrobium* W1-1B swarmer cells exhibited a positive chemotactic response toward the respective substrates. The data were statistically analyzed using a Kruskal-Wallis one-way ANOVA analysis on ranks, incorporating Dunnett’s test when there were equal replicates for all cases and Dunn’s test when the number of replicates differed (SigmaStat, Jandel Scientific, San Rafael, CA). From the data, concentration-response curves were constructed which are an illustration of the degree of response to various concentrations of the potential attractant. Figures 28, 29, and 30 show
Figure 28. Concentration response curves of *Hyphomicrobium* W1-1B cells grown with methylamine to methylamine (A), dimethylamine (B), and trimethylamine (C).
Figure 29. Concentration response curves of *Hyphomicrobium* W1-1B cells grown with dimethylamine to methylamine (A), dimethylamine (B), and trimethylamine (C).
Figure 30. Concentration response curves of *Hyphomicrobium* W1-1B cells grown with trimethylamine to methylamine (A), dimethylamine (B), and trimethylamine (C).
representative data on the chemotactic response of methylamine-, dimethylamine-, and trimethylamine-grown cells, respectively. From these concentration-response curves, the relative peak response can be determined. The relative peak response is the concentration of attractant tested at which the highest ratio of cells per capillary tube with attractant to cells per capillary tube with no attractant (the control) is obtained. Another value, the threshold concentration, was also calculated to assist in describing the chemotactic response. The threshold concentration is defined as the lowest concentration at which a detectable response occurs (Mesibov et al., 1973). Tables 6, 7, and 8 summarize representative chemotaxis data for the experimental conditions tested.

Arginine and methanol were tested as possible attractants with methylamine, dimethylamine, and trimethylamine-grown cells. Statistical analysis using a Kruskal-Wallis one-way ANOVA analysis on ranks (SigmaStat, Jandel Scientific) indicated that neither arginine nor methanol elicited a chemotactic response from the Hyphomicrobium W1-1B swarmer cells. The raw data from the competition experiments were also tested using a Kruskal-Wallis one-way ANOVA analysis on ranks (SigmaStat, Jandel Scientific). Statistical analysis indicated that no chemotactic response was elicited under any of the competition assay conditions tested.

When Hyphomicrobium W1-1B was grown with methanol as the sole carbon and energy source, nascent cells were not motile. Addition of various concentrations of methanol to wet mounts of motile, swarmer cells grown on methylamine did not inhibit
Table 6. Compilation of chemotaxis data from *Hyphomicrobium* W1-1B grown with methylamine as the sole carbon and energy source.

<table>
<thead>
<tr>
<th>Attractant</th>
<th>Ratio</th>
<th>Threshold conc. (mM)</th>
<th>Peak response (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM of attractant in tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$</td>
<td>$10^{-4}$</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>Methylamine</td>
<td>1.4</td>
<td>5.2</td>
<td>17</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>1.6</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>1.3</td>
<td>1.7</td>
<td>1.8</td>
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Table 7. Compilation of chemotaxis data from *Hyphomicrobium* W1-1B grown with dimethylamine as the sole carbon and energy source.

<table>
<thead>
<tr>
<th>Attractant</th>
<th>Ratio</th>
<th>Threshold conc. (mM)</th>
<th>Peak response (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM of attractant in tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-2}$</td>
<td>$10^{-1}$</td>
<td>$10^{0}$</td>
</tr>
<tr>
<td>Methylamine</td>
<td>1.2</td>
<td>3.7</td>
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<tr>
<td>Trimethylamine</td>
<td>0.34</td>
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Table 8. Compilation of chemotaxis data from *Hyphomicrobium* W1-1B grown with trimethylamine as the sole carbon and energy source.

<table>
<thead>
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<th>Attractant</th>
<th>Ratio</th>
<th>Threshold conc. (mM)</th>
<th>Peak response (mM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mM of attractant in tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-2}$</td>
<td>$10^{-1}$</td>
<td>$10^{0}$</td>
</tr>
<tr>
<td>Methylamine</td>
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<td>19.1</td>
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<tr>
<td>Trimethylamine</td>
<td>4.7</td>
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</tbody>
</table>
motility, nor did the addition of supernatant from methanol-grown cells to wet mounts of motile methylamine-grown cells inhibit motility. Likewise, addition of supernatant from methylamine-grown cells to wet mounts of non-motile methanol-grown cells did not induce motility. All examples were observed for 15 -30 minutes to see if motility was affected.

Attempts were made to anchor swarmer cells of Hyphomicrobium W1-1B to tunnel slides using various concentrations of the monoclonal antibody prepared against flagella of R. meliloti 7201. However, the monoclonal antibody encouraged the swarmer cells to clump together rather than become attached to the tunnel slide. When swarmer cells were added to the tunnel slides in the absence of monoclonal antibody, the swarmer cells attached to the tunnel slide so that cellular rotation could be observed. The majority of the cells rotated in a clockwise direction, indicating that the direction of flagellar rotation was counterclockwise. Some cells were observed to temporarily cease all rotation. A few cells rotated in a counterclockwise direction, indicating clockwise flagellar rotation. Addition of various concentrations methylamine did not alter the direction of flagellar rotation. Addition of methanol did not inhibit or alter the direction of flagellar rotation.

Electron microscopy of Hyphomicrobium W1-1B flagella preparations indicated that the preparations were enriched with flagella (Figure 31). Analysis of the flagella preparations using SDS-PAGE indicated that two proteins, of 39,000 $M_r$ and 41,000 $M_r$, were associated with the flagella (Figure 32). Both of the proteins showed cross-reactivity on Western blots to the monoclonal antibody prepared against R. meliloti
Figure 31. Transmission electron micrograph of *Hyphomicrobiurn* W1-1B flagella preparation (A). the arrow indicates a hook (B). Bars = 100 nm
Figure 32. SDS-PAGE of *R. meliloti* 7201 flagella preparation (Lane 1) and *Hyphomicrobium* W1-1B flagella preparation (Lane 2).
7201 flagella, further indicating that the two proteins were associated with the flagella (Figure 33).

Using the organisms tested, the monoclonal antibody showed cross-reactivity only with *R. meliloti* 7201, *Hyphomicrobium* W1-1B, and *Bradyrhizobium japonicum* denatured flagella (Figure 34). This indicates that there is some epitope homology among the flagella of these three organisms.

Flagella prepared from cells grown with methanol and from cells grown with methylamine were also analyzed using SDS-PAGE. Although the methanol-grown cells were non-motile, the same two proteins were detected in the flagella preparation as in the flagella prep from methylamine-grown cells. More of the 39,000 *M*₉ protein was present in the methanol-grown flagella preparation and more of the 41,000 *M*₉ protein was present in the methylamine-grown flagella preparation (Figure 35). Western blots of the proteins from the methanol-grown flagella preparations cross-reacted with the monoclonal antibody to *R. meliloti* 7201 flagella in the same manner as the proteins from the methylamine-grown flagella preps (Figure 36).

Analysis of the 39,000 *M*₉ protein and the 41,000 *M*₉ protein using Schiff's staining technique (Figure 37) and enzyme immunoassay (Figure 38) indicated that neither protein was glycosylated.

Protein fingerprints were prepared of both the 39,000 *M*₉ protein and the 41,000 *M*₉ protein of *Hyphomicrobium* W1-1B and the flagellin protein of *R. meliloti* RMB7201 flagella preparations using three proteases. Digestion of the proteins with
Figure 33. Western blot of *Hyphomicrobium* W1-1B flagella preparation indicating the two flagella-associated proteins (Lane 1) and *R. meliloti* 7201 flagella preparation (Lane 2).
Figure 34. Western blot of flagella preparations from several bacteria using monoclonal antibody prepared against flagella from *R. meliloti* 7201 to determine the specificity of the monoclonal antibody. Lane 1 - *R. meliloti* 7201; Lane 2 - *Hyphomicrobium* W1-1B; Lane 3 - *Pseudomonas aeruginosa*; Lane 4 - *Agrobacterium tumefaciens*; Lane 5 - *Bradyrhizobium japonicum*; Lane 6 - M91-3.
Figure 35. SDS-PAGE of *Hyphomicrobium* W1-1B flagella preparations from methylamine-grown cells, Lane 1; and methanol-grown cells, Lane 2. Equal amounts of protein were added in each lane.
Figure 36. Western blot of *Hyphomicrobium* W1-1B flagella preparations from methylamine-grown cells, Lane 1; and methanol-grown cells, Lane 2.
Figure 37. Schiff's stained SDS-PAGE to identify glycosylated proteins. Lane 1 - *R. meliloti* 7201 flagella preparation; Lane 2 - *Hyphomicrobium* W1-1B flagella preparation; Lane 3 - transferrin, positive control; Lane 4 - creatinase, negative control.
Figure 38. Immunodetection assay to identify glycosylated proteins. Lane 1 - *Hyphomicrobium* W1-1B flagella preparation; Lane 2 - transferrin, positive control; Lane 3 - creatinase, negative control
2% alkaline protease resulted in digestion of all proteins to peptide fragments of 8000 Da or less (Figure 39). Digestion of proteins with 10% Endoproteinase Lys-C resulted in incomplete digestion of all three proteins but still produced a protein fingerprint pattern on silver-stained gels and Western blots (Figure 40). Variations such as increasing the protease concentration (up to 20%) and extending the time of the run in the stacking gel by reducing the current to 10 mA were not successful in obtaining complete digestion of the initial proteins. A 5% concentration of Endoproteinase Glu-C produced complete digestion of all three initial proteins (Figure 41).

Protein fingerprints from Lys-C and Glu-C digests as visualized on silver-stained gels and Western blots were statistically analyzed using the one-sided Fisher's Exact Test (Statxact), based on the distribution of x which is hypergeometric under the null hypothesis of independence. In this case, the null hypothesis was independence of the frequency of zones in the two patterns being compared, or that the data were generated by chance assignments. The alternative hypothesis was that the proportion of matches was larger than expected by chance, thus there was a degree of congruence that was larger than expected by chance. The P value generated was the "total probability on a random basis of x being greater than the observed value" as indicated by Pinder and Gratzer (1972). This value is conventionally compared with 0.05 but Pinder and Gratzer (1972) advocated comparing the P value with 0.01.

For this experiment, the P values were compared with both 0.05 and 0.01. For the conditions tested, only one situation, a Western blot of an Endoproteinase Glu-C
Figure 39. Protein fingerprints of the two flagella-associated *Hyphomicrobium* W1-1B proteins and *R. meliloti* 7201 flagella preparation after digestion with alkaline protease. A is the SDS-PAGE and B is the Western blot. Lane 1 - 41,000 Mr protein from *Hyphomicrobium* W1-1B; Lane 2 - 39,000 Mr protein from W1-B1; Lane 3 - *R. meliloti* 7201.
Figure 40. Protein fingerprints of the two flagella-associated *Hyphomicrobium* W1-1B proteins and *R. meliloti* 7201 flagella preparation after digestion with Endoproteinase Lys-C. A is the SDS-PAGE and B is the Western blot. Lane 1 - 41,000 \( M_r \), protein from *Hyphomicrobium* W1-1B; Lane 2 - 39,000 \( M_r \), protein from W1-B1; Lane 3 - *R. meliloti* 7201.
Figure 41. Protein fingerprints of the two flagella-associated *Hyphomicrobium* W1-1B proteins and *R. meliloti* 7201 flagella preparation after digestion with Endoproteinase Glu-C. A is the SDS-PAGE and B is the Western blot. Lane 1 - 41,000 $M_r$ protein from *Hyphomicrobium* W1-1B; Lane 2 - 39,000 $M_r$ protein from W1-B1; Lane 3 - *R. meliloti* 7201.
digest, had a P value < 0.01, indicating rejection of the null hypothesis (Table 9). All but one of the conditions tested had a P value < 0.05 (Table 9). For these pairs in which independence was rejected when P < 0.05, a 90% confidence interval for the common odds ratio was calculated. The 90% confidence level was chosen so that the interval failed to contain 1.0 (signifying independence) if and only if independence is rejected using a one-sided Fisher’s Exact Test. Pairs with confidence intervals further from 1.0 displayed stronger agreement than pairs with confidence intervals closer than 1.0. Based on these confidence intervals (Table 9), there is some congruence between the two flagellin proteins from *Hyphomicrobium* W1-1B indicating that the proteins are similar but not identical.

**Discussion**

*Hyphomicrobium* W1-1B exhibited a positive chemotactic response toward methylamine, dimethylamine, and trimethylamine. The peak response, or concentration of attractant at which the most bacteria were present in the capillary tube, was 1 mM of attractant for all conditions tested, except for peak responses at 10 mM and 0.1 mM of methylamine-grown cells to methylamine or dimethylamine, respectively. These data indicated that methylamine-grown swarmer cells of *Hyphomicrobium* W1-1B were least sensitive to methylamine as an attractant and most sensitive to dimethylamine as an attractant. In the cases of the dimethylamine and trimethylamine-grown cells, the similarity among the peak responses for all attractants
Table 9. Statistical analysis of protein fingerprints obtained using different proteases and visualizing the fingerprints using either SDS-PAGE or Western blots for the 39,000 $M_r$ and 41,000 $M_r$ flagellin proteins of *Hyphomicrobium* W1-1B using Fisher's Exact Test.

<table>
<thead>
<tr>
<th>Type of endoproteinase treatment and visualization technique</th>
<th>P value from Fisher's Exact Test</th>
<th>Confidence Interval$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-C gel</td>
<td>0.081</td>
<td></td>
</tr>
<tr>
<td>Glu-C Western blot 1 $^b$</td>
<td>&lt;0.001</td>
<td>0.004 - 0.165</td>
</tr>
<tr>
<td>Glu-C Western blot 2</td>
<td>0.052</td>
<td>0.012 - 1.018</td>
</tr>
<tr>
<td>Lys-C gel 1</td>
<td>0.010</td>
<td>0.014 - 0.592</td>
</tr>
<tr>
<td>Lys-C gel 2</td>
<td>0.049</td>
<td>0.015 - 0.993</td>
</tr>
<tr>
<td>Lys-C Western blot</td>
<td>0.026</td>
<td>0.032 - 0.801</td>
</tr>
</tbody>
</table>

$^a$ = A 90% confidence interval for the common odds ratio was calculated when the P value was $\leq 0.05$.

$^b$ = Data for replicates of some treatments are reported.
tested suggested that the chemotactic response was not inducible. While not as uniform in the peak response, the chemotactic response for methylamine-grown cells unaffected by induction because a statistically significant chemotactic response was observed with all three attractants tested.

The threshold concentrations are lowest for dimethylamine as an attractant in dimethylamine-grown cells and for trimethylamine as an attractant in trimethylamine-grown swarmer cells. This suggests a conditioning in the swarmer cells whereby a lower concentration of the attractant which also served as the growth substrate elicits a chemotactic response. *Hyphomicrobium X* has been shown to have an uptake system for methylamine which is inducible (Brooke and Attwood, 1984) and may explain the conditioning seen based on the threshold concentrations for dimethylamine- and trimethylamine-grown cells. However, the threshold concentration for methylamine as an attractant in methylamine-grown cells was higher than for the other attractants.

Swarmer cells under competition experimental conditions did not exhibit a statistically significant chemotactic response regardless of the carbon source used to grow the swarmer cells. These data indicated that the same receptor was involved in the recognition of methylamine, dimethylamine, and trimethylamine as an attractant.

Arginine is a classical chemoattractant and can be utilized by *Hyphomicrobium W1-1B* as the sole nitrogen source but not the sole carbon source. Therefore, arginine was used as a possible chemoattractant for *Hyphomicrobium W1-1B* to determine whether nitrogen sources could be recognized as attractants. However, the results were
inconclusive because arginine did not elicit a chemotactic response in *Hyphomicrobium* swarmer cells.

The ability of the swarmer cells to tether to glass slides in the absence of monoclonal antibodies is not unexpected. Swarmer cells from *Hyphomicrobium ZV580* have been reported to sorb reversibly to glass cover slips with the attachment possibly enhanced by an adhesin having the properties of a glycoprotein or a peptidopolysaccharide (Moore and Marshall, 1981). Moore and Marshall (1981) suggested that the flagella of the tethered cells were involved in transporting the adhesin from the cells to the glass surface. Some potentially pathogenic bacteria rely upon the flagellum as a virulence factor, possibly aiding in attachment to tissues. For instance, flagella from *Pseudomonas aeruginosa* have been shown to contribute to virulence in burn wounds (Drake and Montie, 1988). In addition, motility in *Helicobacter pylori* has been shown to have a correlation with the colonization of tissue resulting in gastritis (Eaton et al., 1989). All strains of *Hyphomicrobium* tested to date are non-pathogenic (Famurewa et al., 1983) but the ability of the flagella to aid in the attachment of *Hyphomicrobium* cells to surfaces may enable the organism to form biofilms, possibly as a survival strategy or a preferred condition for growth. Although the specific means of attachment is currently unknown, the demonstrated ability of *Hyphomicrobium W1-1B* to attach to glass surfaces may explain the prevalence of appendaged organisms on the biofilm-covered slides suspended in water wells.
Video microscopy observations indicated that *Hyphomicrobium* W1-1B flagella rotated predominantly in a counterclockwise direction, but clockwise rotation and temporarily ceasing rotation were not uncommon. If the swarmer cells are capable of all three of these responses, some change in the direction of rotation would be expected when an attractant were introduced. The experiments designed to determine the direction of rotation of the flagella under chemotaxis conditions produced inconclusive results. Once the cells are attached to a surface, the ability to change the direction of rotation even in the presence of an attractant may be altered. Investigation into the regulation of the chemotactic response in tethered vs. free swarmer cells is necessary for further elucidation.

The flagella of *Hyphomicrobium* W1-1B are composed of at least two proteins, 39,000 $M_r$ and 41,000 $M_r$. Unlike three of the flagellin proteins contained in *Halobacteria* flagella (Wieland et al., 1985), the flagellin proteins of *Hyphomicrobium* W1-1B were not found to be glycosylated. Besides glycosylation, other modifications of the flagellin proteins have been described. For instance, the glycosylated *Halobacteria* flagellins are also sulfated (Wieland et al., 1985). A report of phosphorylated flagellin at tyrosine residues in *Pseudomonas aeruginosa* has also been published but the function of the phosphorylation has not yet been described (South et al., 1994). Evidence also suggests that type a and type b flagellins from *P. aeruginosa* are modified in a yet unknown fashion, and that the modified flagellin is the predominant form present in the motile cells (S. Wahl, Dept. of Microbiology, The
Ohio State University, Columbus, OH 43210). Except for glycosylation, no further investigations to detect other modifications of the *Hyphomicrobium* flagellin proteins were conducted.

Qualitative examination of SDS-PAGE and Western blots indicated that more of the 39,000 $M_r$ protein was present in the methanol-grown cells and more of the 41,000 $M_r$ protein was present in the methylamine-grown cells. It may be possible, then, that the non-motility of nascent cells when *Hyphomicrobium* W1-1B is grown with methanol is related to the structure of the flagella. It would be interesting to obtain mutants capable of production of only one or the other flagellin to determine if both flagellins are necessary for motility.

For the protein fingerprinting, when treated with Endoproteinase Lys-C complete digestion of the initial protein was not accomplished. Therefore, the Endoproteinase Lys-C data may not be conclusive. Complete digestion of the initial proteins was accomplished with Endoproteinase Glu-C, and the results from the statistical calculations indicated that the two flagellin proteins from *Hyphomicrobium* W1-1B are similar but not identical. Protein fingerprinting comparing the 57,000 $M_r$ and the 56,000 $M_r$ proteins from *Helicobacter pylori* flagella also indicated that the two flagellin proteins were similar but not identical (Kostrzynska et al., 1991). The fingerprints obtained from the Western blots were statistically more similar than the fingerprints obtained from SDS-PAGE. This suggests a higher degree of homology between the epitope regions for the monoclonal antibody of both of the flagellin
proteins.

In conclusion, this is the first description of chemotaxis and flagellar structure in the methylo trophic, appendaged organism *Hyphomicrobium* W1-1B and involving the elucidation of the chemotactic response and assembly of the flagella in *Hyphomicrobium* spp. Like *Caulobacter* spp., the flagella of *Hyphomicrobium* W1-1B are composed of multiple flagellin proteins. The ability of *Hyphomicrobium* W1-1B flagella to cross-react with the monoclonal antibody against *R. meliloti* 7201 flagella provides a useful and sensitive tool for detection of the two flagellin proteins of *Hyphomicrobium* W1-1B. Further experiments involving the spatial and temporal regulation of flagellin synthesis and assembly as well as motility could begin with identification of the genes responsible for production of the two flagellin proteins.
CONCLUDING REMARKS

While the work with *Hyphomicrobium* W1-1B included the first reports describing the chemotaxis and structure of the flagella of *Hyphomicrobium*, many more questions pertaining to the motility and the flagellin proteins remain unanswered. This investigation only addressed the motile, swarmer cell population of *Hyphomicrobium* W1-1B. Investigations pertaining to the rest of the morphologically differential lifecycle of *Hyphomicrobium* spp., including regulation of differentiation and temporal production of proteins, has not yet been reported.

The chemotactic response was not inducible although there was variation in the threshold concentrations. This variation may be related to the uptake system that *Hyphomicrobium* spp. have for methylamine. To determine whether the threshold concentration and uptake systems are linked, transport-negative mutants could be used to see if a chemotactic response is exhibited in the absence of substrate uptake.

Two proteins were associated with the flagella of *Hyphomicrobium* W1-1B. It is not known whether the flagellar filaments of *Hyphomicrobium* spp. contain these proteins in stoichiometric amounts such in the case of the heterodimer subunits of *Rhizobium meliloti* or in various amounts with distinct spatial locations within the
filament as in *Caulobacter crescentus*. One way to study the composition of the flagellar filaments would be to isolate mutants which produce only one type of flagellin protein. Analysis of the structure of the whole flagella and motility of these mutants may indicate whether the proteins require assembly in a specific order or proportion for motility.

The ability of the *Hyphomicrobium* W1-1B flagellin proteins to cross-react with the monoclonal antibody prepared against *Rhizobium meliloti* RMB7201 flagella is also intriguing. Protein fingerprinting indicated that the flagellins from the two organisms were not closely related, yet the epitope for this monoclonal antibody is shared. Determination of the amino acid composition of the *Hyphomicrobium* W1-1B and *R. meliloti* flagellins would be useful in further examining the relatedness of these proteins to each other and to flagellin proteins from other organisms. Sequencing of the N-terminal amino acids has been used to compare the conserved areas in two or more flagellin proteins. Sequencing would be useful here to (i) determine the relatedness of the two *Hyphomicrobium* W1-1B flagellins and (ii) determine the relatedness of the *Hyphomicrobium* W1-1B flagellins to *R. meliloti*. Further studies addressing the shared epitopes of the *Hyphomicrobium* W1-1B, *R. meliloti* RMB7201, and *Bradyrhizobium japonicum* flagellins would also be interesting. Use of immunogold labeling could be used to determine the location of the epitopes for the monoclonal antibody on the flagellins and to determine if the epitopes on whole flagella are exposed to the surface or hidden within the intact filament.
Ultimately, studies involving the temporal regulation of the flagellin proteins would require the identification of the genes responsible for encoding both proteins. This information would lend insight into the regulation of cell cycle dependent events which would be instrumental in further studies concerning the lifecycle of *Hyphomicrobium* spp.
REFERENCES


