Neutrophilic, Microaerophilic Fe(II)-Oxidizing Bacteria are Ubiquitous in Aquatic Habitats of a Subtropical Australian Coastal Catchment (Ubiquitous FeOB in Catchment Aquatic Habitats)

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Neutrophilic, Microaerophilic Fe(II)-Oxidizing Bacteria are Ubiquitous in Aquatic Habitats of a Subtropical Australian Coastal Catchment (Ubiquitous FeOB in Catchment Aquatic Habitats)

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We examined the abundance and distribution of neutrophilic, microaerophilic Fe(II)-oxidizing bacteria (FeOB) in aquatic habitats of a highly weathered, subtropical coastal catchment where Fe biogeochemistry is of environmental significance. Laboratory cultivation and microscopy indicated that stalked Gallionella and sheathed Leptothrix-like FeOB were present in microbial mats associated with a circumneutral-pH, groundwater seep and stream-bank surface sediment, whereas unicellular FeOB were widespread in surface and subsurface waters, including a seep, shallow stream and estuary-adjacent groundwater. Direct Gallionella-specific PCR detected dominant bacterial members related to Sideroxydans paludicola (95% sequence identity, SI) and Gallionella capsiferriformans (96% SI) in the seep microbial mat. TGGE analysis indicated that the most common FeOB in water enrichment cultures were related to S. lithotrophicus (96% SI). The ubiquity of FeOB in Poona catchment aquatic habitats suggests bacterial Fe(II) oxidation is integral to catchment Fe biogeochemistry.

Keywords Iron biogeochemistry, iron-oxidizing bacteria, Gallionella, Sideroxydans, subtropical Australia

INTRODUCTION

Iron (Fe) is one of the most abundant redox-active elements in the Earth’s crust. In aquatic habitats at circumneutral pH, Fe undergoes active redox reactions between Fe(II) and Fe(III). Tightly linked Fe redox reactions at oxic–anoxic interfaces can be substantially driven by microbial processes, and microbial Fe redox cycling has been proposed for a variety of freshwater and marine environments such as groundwater Fe seeps, wetland plant rhizospheres and deep-sea hydrothermal vents (Sobolev and Roden 2002; Weiss et al. 2003; Blöthe and Roden 2009; Emerson 2009).

Microbial Fe cycling is of environmental significance given the critical influence Fe cycling exerts on the behavior of organic and inorganic compounds in aquatic systems (Roden and Emerson 2007). Lovley and co-workers have highlighted the significance of microbial Fe(III) reduction in subsurface environments at circumneutral pH (Lovley and Phillips 1988; Lovley 1993; Lovley 2006). Mechanisms of microbial Fe(III) reduction were also investigated with representative Geobacter and Shewanella strains (Nevin and Lovley 2002).

However, the significance of microbial Fe(II) oxidation in circumneutral environments remains poorly understood, and progress in understanding associated mechanisms and microorganisms has been slow. This is, in part, due to difficulty in establishing laboratory microcosms at circumneutral pH because of the instability of Fe(II)/(III) compounds, and assumptions that abiotic processes dominate Fe cycling under circumneutral conditions.

Morphologically distinctive bacteria, including stalked Gallionella and sheathed Leptothrix, as well as hyphal, budding Pedobacterium, have previously been the genera commonly identified as neutrophilic, microaerophilic Fe- (and/or Mn-) oxidizing (and/or depositing) bacteria (FeOB) (Pringsheim 1949; Corstjens et al. 1992; Larsen et al. 1999; Pedersen and Hallbeck 2008). In recent decades, a novel group of unicellular FeOB
represented by Sideroxydans was identified in Fe(II)-containing groundwater, freshwater wetlands and plant roots, and deep-sea marine environments (Emerson and Moyer 1997; Emerson et al. 1999; Emerson and Moyer 2002; Edwards et al. 2003; Blöthe and Roden 2009; Wang et al. 2009).

Novel FeOB commonly occur at oxic–anoxic interfaces of Fe-rich circumneutral environments where rust-colored Fe oxide precipitate is distinctive. Despite frequent observations of abundant stalks and sheaths resembling Gallionella and Leptothrix, Emerson and Revsbech (1994) suggested unicellular bacteria may comprise a larger population in the Fe(II)-oxidizing microbial mat community.

Current knowledge of the ecology and microbial diversity of FeOB in aquatic environments is limited. Known FeOB are phylogenetically diverse, and the lack of knowledge of genes involved in Fe(II) oxidation restricts degenerate primer design for group-specific detection of novel FeOB from environmental samples (Emerson et al. 2007; Weiss et al. 2007).

Thus, culture-based studies combined with high-throughput molecular phylogenetic methods such as Terminal Restriction Fragment Length Polymorphism (TRFLP), Denaturation or Temperature Gradient Gel Electrophoresis (DGGE/TGGE), cloning and sequencing, are currently of importance (Sudek et al. 2009; Wang et al. 2009; Blöthe and Roden 2009; Emerson et al. 2007). However, recent studies of Fe(II) oxidation biogeochemistry and related microbial communities in aquatic habitats tended to focus on specific Fe(II)-rich water bodies characterized by abundant microbial mats associated with Fe oxide precipitates (Blöthe and Roden 2009; Duckworth et al. 2009; Sudek et al. 2009; Bruun et al. 2010). No studies have reported on microbial Fe(II) oxidation in a diverse aquatic environment including surface and subsurface waters.

This study aimed to examine the abundance and distribution of FeOB in a range of aquatic habitats in the subtropical catchment of Poona Creek, South-East Queensland (SE Qld, Australia). Poona catchment features highly weathered soils and localized Fe concretions (Löhr et al. 2010a, b). It comprises a plantation-forested coastal catchment that drains into the UNESCO-listed Great Sandy Strait, an environmentally sensitive estuarine habitat of international significance. In recent years, active forestry practices such as harvesting and replanting have occurred within the catchment area (Lin et al. 2010).

Previous research in tropical and subtropical forests suggests plantation conversion may elevate soil organic carbon availability, and thus stimulate biological or abiotic Fe reduction and dissolution under flooded conditions (Chacon et al. 2006; Fang et al. 2009). Localized Fe mobilization together with contaminant species from soils and sediments into aquatic environments may negatively impact water quality through biofouling, and cause microbially influenced corrosion of associated industrial metal equipment (Chamritski et al. 2004; Stuetz and McLaughlan 2004; Ray et al. 2009).

Transport of Fe and associated compounds into estuarine and marine environments may also provide limiting nutrients for blooms of the potentially toxic cyanobacterium Lyngbya majuscula (Ahern et al. 2003, 2005, 2008). Given that microbial activities play an important role in Fe dissolution and mobilization (Stemmler and Berthelin 2003; Fakih et al. 2008), investigation of Fe biogeochemistry-associated bacterial populations is necessary.

We examined the abundance and distribution of FeOB in Poona catchment aquatic habitats using a variety of gradient culture techniques in combination with microscopy and 16S rRNA-based molecular phylogenetic techniques. The results will contribute to understanding bacterial involvement in subtropical coastal catchment Fe biogeochemistry, expanding our knowledge of the ecology of FeOB in aquatic environments.

MATERIALS AND METHODS

Field Site Description and Sampling

Poona catchment (ca. 100 km²) is located 300 km north of Brisbane, on the Fraser Coast of SE Qld, Australia (Figure 1A). Local annual average rainfall is 1270 mm, occurring mostly during the warmer months (October–March). Mean monthly

FIG. 1. Location of Poona catchment study area (A) and sampling sites (B) (color figure available online).
maximum temperatures range from 21.5 °C in July to 30.2 °C in December (Löhr et al. 2010b). Water flow in Poona catchment is ephemeral in the upper reaches but perennial in the lower reaches. Pinus plantation dominates 56 km² of the catchment, with native vegetation buffer zones along waterways adjacent to poorly drained soils. Fe(II) concentration in Poona catchment aquatic systems ranges from <0.1 to >10 mg L⁻¹, reaching values as high as 25 mg L⁻¹ in shallow groundwater near the estuary (data not shown). Several seeps occur mid-catchment at Pappin’s Bridge (PB), continuously fed by Fe(II)-containing groundwater and characterized by copious microbial mats of reddish-brown color. Similar mat material was found on surface sediments of the estuarine transect (TS) and the streambank at Poona Creek South (PCS), but not at Water Point 19 (WP) (Figure 1B).

To target stalk-forming Gallionella, microbial mat material was aseptically collected from a PB groundwater-fed seep for laboratory cultivation, microscopy and molecular biological analysis. Mat samples were also collected from surface sediments at PCS streambank and TS estuary-adjacent transect for microscopy. Water samples for biological analysis were aseptically collected from the slowly flowing, mat-associated seep (PB), shallow stream characterized by foaming (WP), and estuary-adjacent groundwater extracted from a shallow monitoring well (TS, Figure 1B).

Biological samples were stored in sterile Sarstedt specimen containers (for mat material) or autoclaved glass bottles (for water), transported on ice to the laboratory and processed for bacterial cultivation within 24 h upon arrival. Water physico-chemical parameters including temperature, pH, redox potential, electrical conductivity and dissolved O₂ were measured at PB seep, WP stream and TS groundwater using a calibrated TPS 90-FMLV multiple field analyzer. For analysis of carbon substrates and Fe(II), as well as sulfate, sulfide and nitrate, water samples were retrieved from the three sites, stored in acid-washed HDPE plastic bottles, preserved as described previously (Eaton et al. 2005), transported on ice to the laboratory and stored at 4 °C before analysis.

To examine the occurrence of stalk-forming Gallionella in situ, sterilized (160 °C, 2h) mild steel slides (50 × 22 mm) were exposed at PB seep, WP stream and TS groundwater. The slides were vertically positioned at a depth of 10–20 cm below the water surface and retrieved after 10 d.

**Hydrochemistry**

Water samples were analyzed for total dissolved organic carbon using a Shimadzu TOC-5000A TOC Analyzer; bicarbonate (alkalinity), Fe species, sulfate and nitrate using a SEAL-AQ2 Discrete Analyzer; sulfide using the methylene blue method (Eaton et al. 2005). Results are presented as arithmetic means.

**Bacterial Cultivation, Enumeration and Enrichment**

Three variations of FeS gradient medium including liquid, semi-solid and biphasic slant media were prepared and used as described previously (Verran et al. 1995; Atlas 2004; Emerson and Floyd 2005; Hanert 2006). All gradient media contained an FeS colloid (Hanert 2006) overlaid with bicarbonate-buffered Modified Wolfe’s Mineral Medium (MWMM) supplemented with vitamins solution (Widdel and Bak 1992) and bubbled with filter-sterilized CO₂.

Screw-capped 16 × 125 mm disposable tubes were aseptically dispensed with 0.5 mL FeS colloid and 9 mL MWMM for liquid gradient medium; 2 mL FeS colloid (1% agarose, Sigma–Aldrich) and 8 mL gel-stabilized MWMM (0.15% agarose) for semi-solid gradient medium; 6 mL FeS colloid (3% agarose) and 2 mL MWMM for biphasic slant gradient medium. Media were freshly prepared, stored under an anoxic atmosphere for a minimum of 4 h and inoculated within one week. Opposing O₂ and Fe(II) gradients that developed in the liquid or gel-stabilized semi-solid overlay allowed FeOB to grow at the oxic–anoxic interface. A sterile glass rod was placed in each tube of the liquid gradient medium during initial enrichment to increase the potential surface area for bacterial growth.

Bacterial cultivation and enrichment were performed by directly inoculating 0.5, 0.1 and 0.01 mL aliquots of suspended mat material (PB) and water (PB, WP and TS) into the three gradient media. For enumeration, samples were also diluted over the range 10⁻³ to 10⁻⁶ using MWMM and inoculated into a 1:10 dilution-to-extinction series in liquid gradient medium. Approximate bacterial density was calculated from the highest dilution showing positive growth. Sterile distilled water was used as an uninoculated control. Inoculated media were incubated in the dark at 25 °C for two (liquid gradient medium) or four weeks (semi-solid gradient medium).

Presumptive positive growth was recorded based on the observation of colonies in liquid medium, or a banding pattern in semi-solid and biphasic slant media. The presence of bacterial cells was confirmed using light microscopy (1000× total magnification). Single colonies from liquid or semi-solid enrichment cultures were transferred into fresh media for subculturing. Due to a low cell yield in the subculture, cells were collected from sessile stalk-forming Gallionella-like colonies of the initial mat enrichment culture in liquid gradient medium. The stalk-forming Gallionella-like FeOB was not recoverable from subculture.

For water enrichment cultures, cells of the fourth subculture were collected from the Fe(II) oxidation-band in semi-solid gradient medium. Cell material was heated at 70 °C in a water bath for 12 min, and centrifuged at room temperature for 5 min to remove agar from cell suspensions (Emerson and Moyer 1997). Cell pellets were washed twice in phosphate buffered saline (pH 6.8) and stored at −80°C before molecular biological analysis.

**Sample Preparation for Microscopy**

A sterile syringe or Pasteur pipette was used to detach single colonies from the tube wall of liquid gradient medium, to obtain 0.5–1.0 mL material from the Fe(II) oxidation band in semi-solid gradient medium and suspended microbial mat, and
microaerophilic Fe(II)-oxidizing bacteria

DNA Extraction and Amplification

Total genomic DNA (gDNA) was extracted from selected samples, that is, PB seep mat material and associated liquid enrichment culture, and PB, TS, WP, TS streamwater and associated semisolid enrichment cultures. A PowerSoil™ DNA isolation kit (MoBio) was used following manufacturers instructions. The gDNA extracts were checked for quality and quantity with 1% (w/v) agarose gel electrophoresis before storage at −20°C prior to molecular biological analysis.

Bacterial primers 341F-GC/907R were used to amplify a 550 bp 16s rDNA fragment from gDNA extracts of enrichment cultures for TGGE analysis (Muyzer et al. 1995). The PCR reaction contained 0.5 U Roche Taq DNA polymerase, 2.5 µL 10× PCR buffer, 3.0 mM MgCl₂, 400 µM dNTP, 6.25 pmol each primer, and 1–2 µL template DNA. Volume was adjusted to 25 µL with sterile Gibco Ultrapure™ DNase/RNase-free distilled water. Acidithiobacillus ferroxydans (kindly provided by J. Plumb) was used as a positive control. PCR amplifications were performed in an Eppendorf Mastercycler S following a touchdown protocol (Muyzer et al. 1995). Final primer extension was carried out at 72°C for 3 min, with 30 total cycles. PCR products of low yield were concentrated with cold ethanol before TGGE analysis.

Gallionella-specific primers 122F/988R were used for detection of Gallionella-related FeOB from environmental samples (Wang et al. 2009).

The PCR reaction was prepared using goTaq Flexi DNA polymerase following manufacturers instructions. Amplification conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, and final extension at 72°C for 10 min (J. Wang, pers. comm.). Nested PCR was performed with 341F-GC/907R using the same PCR reaction and touchdown protocol as described above. A Gallionella sp. DNA sample (kindly provided by J. Wang) was included as a positive control. PCR products were checked for quality and quantity with 1% (w/v) agarose gel electrophoresis.

TGGE-Sequencing and Phylogenetic Analysis

TGGE analysis was performed using the Diagen TGGE system (Düsseldorf, Germany) with horizontal polyacrylamide gels containing 5% (w/v) acrylamide/bis (37.5:1), 8 M urea and 2% (v/v) glycerol in 1× ME electrophoresis buffer (20 mM 4-morpholinepropanesulfonic acid, 1 mM EDTA, pH 8.0). An aliquot of 5 µL PCR product was applied to the gel. After electrophoresis at 300 V for 3.5 h (48–62°C), the gel was silver stained and visible DNA bands were excised using a sterile scalpel blade.

Excised bands were transferred into microcentrifuge tubes containing 30 µL elution buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8.0), and incubated at 4°C overnight. Eluted DNA was concentrated to 10 µL, with 1 µL re-amplified using bacterial primer set 341F/907R and the same PCR reaction and touchdown protocol as described above. Re-amplified DNA was cleaned up using an UltraClean™ PCR clean-up kit (MoBio) and automated DNA sequencing was performed using an ABI 3500 Genetic Analyzer.

Sequence data was manually checked and modified using 4Peaks (A. Griekspoor and Tom Groothuis, http://mekentosj.com), then compared with existing sequences in the GenBank database using BLAST (http://ncbi.nlm.nih.gov/BLAST/Blast). Sequences were automatically aligned with their closest relatives in the Greengene database (Nov, 2008) using the ARB program (Ludwig et al. 2004). Alignment was manually checked, and corrected if necessary. The phylogenetic tree was constructed using the Neighbor-joining algorithm. Sequences were deposited in Genbank with accession numbers HQ117912-HQ117920.

RESULTS

Microbial Mat Characteristics and Water Physicochemical Properties

Microbial mat material that accumulated in the PB seep was attached to rock surfaces or plant residues, with color varying from bright orange-red to dark reddish-brown. A stratified macroscopic structure was observed, with loosely associated slimy material overlaying crusty material (Figure 2). Light microscopy showed both layers contained abundant Gallionella-like twisted stalks and empty Leptothrix-like tubular sheaths, with those from the slimy layer lightly encrusted (Figure 2A), and those from the crusty layer heavily encrusted with rust-colored precipitates (Figure 2B). Gallionella- and Leptothrix-like stalks and sheaths were also present in surface sediment-associated PCS streambank mat material, but not the TS estuary-adjacent transect (data not shown).

Water physicochemical properties are summarized in Table 1. PB seep and WP stream water had a circumneutral pH (5.8–6.4),
FIG. 2. Photograph of PB seep microbial mat. Light micrographs of (A) lightly encrusted stalks and sheaths from surface slimy material; and (B) heavily encrusted stalks and sheaths from deeper crusty material (color figure available online).

and were slightly oxygenated, with redox potentials of 120–212 mV and dissolved O$_2$ of 3.9–5.6 mg L$^{-1}$. By comparison, TS estuary-adjacent groundwater was slightly acidic (pH 4.9), with a lower redox potential (35 mV) and dissolved O$_2$ (0.3 mg L$^{-1}$). The concentrations of Fe(II), bicarbonate and sulfide were substantially higher in TS estuary-adjacent groundwater (6.9, 43.2 and 0.3 mg L$^{-1}$, respectively) than those in the PB seep and WP stream. Dissolved organic carbon (mg L$^{-1}$) at the three sites followed the order WP (24.6) > TS (18.0) > PB (7.1). Sulfate ranged from 4.4 to 5.6 mg L$^{-1}$. Nitrate was below the minimum detection limit (0.03 mg L$^{-1}$).

After 10 d exposure, steel slides retrieved from PB seep and WP stream were coated by a thick layer of reddish-brown mat material that appeared macroscopically homogeneous, while the slide from TS groundwater was free of mat material. Light microscopy showed the presence of *Gallionella*-like stalks and sheaths with mat material deposited on the PB slide. Stalks/sheaths were not observed in mat material deposited on the WP slide, and mat material was not observed in situ at WP.

### Bacterial Cultivation, Enumeration and Enrichment

Stalk-forming *Gallionella*-like FeOB (10$^3$–10$^4$ cells mL$^{-1}$) were recovered only from the PB seep mat material, whereas unicellular FeOB were recovered from all water samples, with the highest densities in TS groundwater (10$^4$–10$^5$ cells mL$^{-1}$), and the lowest in WP stream water (10–10$^2$ cells mL$^{-1}$). Approximately 10$^2$–10$^3$ cells mL$^{-1}$ of unicellular FeOB were recovered from PB seep water.

Bacterial colony characteristics of FeOB were different in the three variations of FeS gradient media which contained similar constituents. Within liquid gradient medium, *Gallionella*-like colonies appeared white and fluffy in the first 2–3 d (Figure 3A), gradually turning a rust color. Sessile *Gallionella*-like colonies tended to slough off the growth surface and did not produce stalks after subculturing. Unicellular FeOB appeared as orange colonies at varying depths in the overlay, tightly attached to the tube wall or glass rod over 3–5 d of incubation (Figure 3B). Individual colonies rarely exceeded 1 mm in diameter, with satellite growth observed around some colonies when >5 d old (Figure 3C).

### TABLE 1

Poona catchment water physicochemical properties (May 2009)

<table>
<thead>
<tr>
<th>Water source</th>
<th>PB</th>
<th>WP</th>
<th>TS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td>17.1</td>
<td>16.8</td>
<td>21.0</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>6.4</td>
<td>5.8</td>
<td>4.9</td>
</tr>
<tr>
<td><strong>Eh (mV)</strong></td>
<td>120</td>
<td>212</td>
<td>35</td>
</tr>
<tr>
<td><strong>DO (mg L$^{-1}$)</strong></td>
<td>5.6</td>
<td>3.9</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>EC (µS cm$^{-1}$)</strong></td>
<td>378</td>
<td>175</td>
<td>360</td>
</tr>
<tr>
<td><strong>Fe$^{2+}$ (mg L$^{-1}$)</strong></td>
<td>2.8</td>
<td>1.0</td>
<td>6.9</td>
</tr>
<tr>
<td><strong>DOC (mg L$^{-1}$)</strong></td>
<td>7.1</td>
<td>24.6</td>
<td>18.0</td>
</tr>
<tr>
<td><strong>HCO$_3^-$ (mg L$^{-1}$)</strong></td>
<td>12.8</td>
<td>0.3</td>
<td>43.2</td>
</tr>
<tr>
<td><strong>SO$_4^{2-}$ (mg L$^{-1}$)</strong></td>
<td>4.4</td>
<td>5.6</td>
<td>5.1</td>
</tr>
<tr>
<td><strong>S$^{2-}$ (mg L$^{-1}$)</strong></td>
<td>0.06</td>
<td>0.05</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>NO$_3^-$ (mg L$^{-1}$)</strong></td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

Eh (redox potential), DO (dissolved O$_2$), EC (electrical conductivity), and DOC (dissolved organic carbon), NO$_3^-$ (measurements below the minimum detection limit).
FIG. 3. Photographs of bacterial colony morphology in liquid (A–C, 3–5 d) and semi-solid gradient media (D, 10 d). (A) White *Gallionella*-like colonies (arrows) attached to tube wall in PB seep mat enrichment culture; (B) rust-coloured unicellular FeOB colonies throughout the liquid overlay of PB seep water enrichment culture; (C) unicellular FeOB colonies showing satellite growth (arrows) in PB seep water enrichment culture; and (D) dense Fe(II) oxidation band resulting from bacterial growth in the semi-solid overlay of WP stream water enrichment culture (color figure available online).

Within semi-solid gradient medium, individual colonies, or a flare-like banding pattern of Fe(II) oxides developed ca. 1 cm above the FeS colloid 3–5 d after inoculation. Over the next 5–10 d, a discrete, dense Fe(II) oxidation band of reddish-brown color formed, displaying a sharp lower, but a diffuse upper, edge (Figure 3D). The semi-solid gradient medium was preferentially used due to higher gDNA extraction yield as compared with the other two media, of which the biphasic slant medium was only used for initial enrichment, and was excluded from subsequent subculturing because of substantially lower microbial biomass production. Bacterial colonies in the biphasic slant medium liquid overlay appeared as a ring attached to the tube wall, with a second ring observed underneath after 3 weeks incubation (data not shown).

**Morphological Studies**

Light microscopy showed the distinctive morphology of stalk-forming *Gallionella*-like FeOB in liquid but not semi-solid enrichment culture of the PB seep microbial mat (Figure 4A). Despite abundant stalks, few apical cells were found attached to stalks (Figure 4B). SEM showed twisted stalks associated with Fe oxide precipitates at different degrees of encrustation, in the absence of apical cells (Figure 4C–E).

Unicellular, curved rods were abundant in liquid enrichment cultures of water samples (Figure 5A). Light microscopy showed bacterial cells approximately 0.3–0.5 \( \mu \text{m} \) in diameter and highly motile within the first few days of cultivation. SEM showed cells devoid of surface encrustation (Figure 5B), as well as cells with globular nanometer-sized Fe oxides sparsely distributed on the surface (Figure 5C), and empty and complete crusts of crystallite Fe oxides (Figure 5D, E). TEM showed that periplasmic Fe oxide deposition was common (Figure 5F). A similar bacterial cell morphology and Fe oxide encrustation pattern were observed with unicellular FeOB in semi-solid enrichment cultures of water samples (Figure 5G–I).

FIG. 4. Light micrographs of (A) abundant stalks from a white *Gallionella*-like colony in PB seep mat liquid enrichment culture; and (B) apical cells (arrows) attached to *Gallionella*-like stalks; and SEM images of (C) twisted stalk lightly encrusted with Fe oxides; (D) stalks heavily encrusted with Fe oxide crystallites; and (D) stalks covered with copious Fe oxide aggregates (color figure available online).
FIG. 5. SEM and TEM images of unicellular FeOB in liquid (A–F, TS groundwater, 7 d) and semi-solid enrichment cultures (G–I, PB seep water, 28 d). (A) Unicellular cells with proximal Fe oxide precipitates; (B) cells associated with Fe oxides but devoid of encrustation; (C) globular Fe oxides deposited on the cell surface; (D) empty crystalline Fe oxide crusts; (E) cells with/without complete Fe oxide crusts; (F) periplasmic deposition; (G) non-encrusted cells associated with agar and Fe oxide precipitates; (H) cells completely encrusted with Fe oxides; and (I) periplasmic accumulation.

In the FeS gradient medium control, Fe oxide precipitation occurred within 2–3 d due to abiotic oxidation. SEM-EDS analysis confirmed Fe and O as the primary elements in both biogenic and abiotic Fe precipitates (data not shown).

**Molecular Biological Analysis**

Liquid enrichment culture of stalk-forming *Gallionella*-like FeOB did not yield sufficient microbial biomass for 16S rDNA-based molecular biological analysis. Direct *Gallionella*-specific PCR with gDNA extracted from the PB seep mat sample detected three dominant bacterial members (GA001-003, Figure 6), with the closest cultivated relatives *Sideroxydans paludicola* (GA001 and GA003, 95% sequence identity, SI) and *Gallionella capsiferriformans* (GA002, 96% SI) (Figure 7).

PCR–TGGE analysis of water enrichment cultures using bacterial primers indicated that the most common unicellular FeOB recovered from all water samples was related to *Sideroxydans lithotrophicus* (PN022, 96% SI). A putatively pure isolate was obtained for PN022 from the PB seep water enrichment culture, demonstrating a single band on a TGGE gel (data not shown). Another *S. lithotrophicus*-related bacterium (PN032, 96% SI) was detected from the WP stream water enrichment culture, whereas a *Gallionella capsiferriformans*-related bacterium (PN013, 96% SI) was detected from the TS groundwater enrichment culture (Figures 6 and 7). Other bacterial members of water enrichment cultures included *Ralstonia pickettii* (PN014, 98% SI), *Burkholderia tropica* (PN019, 100% SI) and *Dyella japonica* (PN024, 98% SI). Direct *Gallionella*-specific PCR with gDNA extracted from water samples yielded no positive results.

FIG. 6. PCR–TGGE of laboratory enrichment cultures and environmental samples. Lane 1 (*Gallionella*-specific primers 122F/988R) and lane 2–4 (bacterial primers 341F-GC/907R) from different gels. Bands of similar migration were excised and sequenced (data not shown).
DISCUSSION

Occurrence and Distribution of FeOB

Laboratory cultivation and microscopy indicated that stalked *Gallionella*- and sheathed *Leptothrix*-like FeOB were present in microbial mats associated with surface freshwater environments including a circumneutral-pH, groundwater-fed seep and streambank surface sediment. In contrast, unicellular FeOB were widespread in surface and subsurface waters, including a seep, shallow stream and estuary-adjacent groundwater. Sufficient Fe(II) and bicarbonate at low redox potential appeared to support high densities of FeOB.

The relative abundance of FeOB in Poona catchment aquatic habitats is comparable to those found in Fe(II)-containing habitats where rapid Fe cycling is known to occur, such as a Danish groundwater seep \((10^3\text{–}10^5\text{ cells mL}^{-1})\), Northern Virginia wetland stream \((8.3 \times 10^5\text{ cells mL}^{-1}\text{ Fe floc, 1–10% of the total population})\), and diverse wetland plant rhizospheres \((5.7 \times 10^5\text{ cells g}^{-1}\text{ root, ca. 1.4% of all rhizosphere bacteria})\) (Emerson et al. 1999; Weiss et al. 2003; Emerson and Weiss 2004; Blöthe and Roden 2009). Although culture-based methods did not provide information on microbial activities, the widespread distribution of FeOB, particularly unicellular species, in Poona catchment aquatic habitats suggests bacterial Fe(II) oxidation is of importance in the catchment Fe biogeochemistry.

During laboratory cultivation, stalked *Gallionella*-like FeOB were observed only in liquid gradient medium inoculated with seep mat material. The *Gallionella*-like culture rapidly produced a large number of stalks within 2–3 d of incubation. Previous research indicates that stalks are not essential for the growth of *Gallionella*, but may act as a holdfast, a template for Fe oxide precipitation, and may protect cells against toxic O2 species formed during rapid abiotic Fe(II) oxidation (Hallbeck et al. 1993; Hallbeck and Pedersen 1995; Suzuki et al. 2011). Hallbeck and Pedersen (1990) demonstrated that *Gallionella ferruginea* can be without stalks when growing exponentially or in low redox potential water, and may lose the stalk-forming ability during laboratory cultivation. This is consistent with our observation that stalked *Gallionella*-like colonies did not produce stalks on subculturing. It is also possible that a non-stalk-producing strain from the enrichment culture outcompeted the stalk-forming strain on subculture.

Despite the abundant biomass morphologically similar to *G. ferruginea*, few apical cells were found attached to *Gallionella*-like stalks in the seep mat liquid enrichment culture (Figure 4). The lack of cells likely precluded the extraction of sufficient gDNA for molecular biological analysis. To generate physiological and phylogenetic data of *G. ferruginea* in several studies, Pedersen and Hallbeck (2008) used a total of 6 kg FeS and 120 L mineral salts solution to prepare approximately 12,000 tubes for a 0.05 g biomass yield. Although we cultivated stalked *Gallionella*-like FeOB from Poona seep mat material containing abundant stalks, *Gallionella*-specific PCR performed on gDNA extracted from the mat material did not detect *G. ferruginea*. However, three dominant bacterial members detected (GA001–003, Figure 6) were related to...
G. capsiferriformans and S. paludicola, neither of which are known to produce stalks.

Gallionella-like stalks have not been observed in laboratory semi-solid enrichment cultures. Blöthe and Roden (2009) reported that Gallionella spp. could not be isolated from seep mat material using semisolid FeS gradient medium, even though Gallionella comprised 33 out 130 clones in a 16S rRNA clone library. Emerson and Moyer (1997) suggested some novel unicellular FeOB may not survive under liquid-culture conditions. These findings demonstrate the limitation of semi-solid gradient media for cultivation of stalk-forming Gallionella, and the limitation of liquid gradient media for unicellular FeOB. Hence, culture-based survey using semi-solid (or liquid) gradient medium alone may substantially underestimate FeOB diversity and abundance.

Gallionella are often associated with slowly flowing, Fe(II)-rich freshwater exposed to O2—typically groundwater seeps, boreholes, ditches and drainages (Hanert 2006). Gallionella habitats commonly have high Fe(II) (5–25 mg L−1), low organic carbon (<12 mg L−1) and dissolved O2 concentrations (0.1–1 mg L−1), and circumboreal pH (6.0–7.6) with sufficient dissolved CO2 (>20 mg L−1) (Hanert 2006). Low temperature (8–16°C) and slow water flow appear prerequisites for stalk formation (Pedersen and Hallbeck 2008). These conditions are comparable to those of the Poona catchment mat-associated seep, except for the higher summer air temperature (>20°C) and dissolved O2 (5.6 mg L−1).

Despite frequent observations of Gallionella-like stalks in oxygenated ditches, drainage pipes and wells, Hallbeck and Pedersen (1990) suggested favourable growth conditions for G. ferruginea exist in low redox potential groundwater. We also recovered a non-stalk-forming G. capsiferriformans-related FeOB from estuary-adjacent groundwater using laboratory cultivation (PN013, Figure 6), although direct Gallionella-specific PCR (Wang et al. 2009) with DNA extracted from groundwater yielded no positive results.

TGGE analysis showed the most common unicellular FeOB recovered from all water samples using semi-solid gradient medium was related to S. lithotrophicus (PN022, 96% SI), with another S. lithotrophicus-related bacterium (PN032, 96% SI) recovered from shallow stream water (Figure 6). Similar to G. capsiferriformans and S. paludicola, S. lithotrophicus is a unicellular FeOB capable of growing on Fe(II) and fixing CO2 (Emerson and Moyer 1997; Weiss et al. 2007). Recently-recognized novel FeOB span several Proteobacteria subdivisions and occur in diverse freshwater and marine environments of wide pH (<4 to >7) and temperature (<10°C to >100°C), (Emerson and Moyer 1997; Emerson et al. 2007; Weiss et al. 2007).

As very few Sideroxydans (S. lithotrophicus and S. paludicola, 95% SI) have been described, our enrichment cultures (PN022 and PN032, 95% SI) may represent novel strains of Sideroxydans. Further characterization of isolate PN022 will establish whether it represents a novel Sideroxydans species, expanding the limited knowledge regarding the ecology, physiology and phylogeny of this genus.

Ralstonia, Burkholderia and Dyella (aerobic bacteria which are often soil-associated) were also recovered from water samples using semi-solid gradient medium (Figure 6) (Coenye and Vandamme 2003; An et al. 2005; Kiratisin et al. 2007). Ralstonia sp. has recently been identified in a Danish freshwater seep microbial mat community in association with tightly-coupled Fe redox cycling (Bruun et al. 2010), whereas Burkholderia and Dyella spp. have been reported capable of biotite weathering and Fe extraction from inorganic sources in forest soil (Uroz et al. 2009). It is uncertain whether these aerobic bacteria are capable of Fe(II) oxidation in situ, and whether they share a competitive or symbiotic relationships with putatively lithotrophic FeOB in enrichment cultures.

Bacterial Fe(II) Oxidation and Fe Biomineralization

Previous observations of Fe oxide precipitation on hydrated Gallionella-like stalks suggest progressive formation of different minerals occurs, including nucleation and precipitation of hematite inside stalk fibers, and subsequent precipitation and accretion of mixed ferrihydrite and goethite on stalk-associated hematite (Hallberg and Ferris 2004). This may account for the macroscopically stratified structure of Poona catchment seep mat material, where encrusted stalks continue to passively absorb Fe(II), which then auto-oxidizes forming a thicker Fe oxide layer deeper in the mat (Figure 2).

SEM analysis of seep mat liquid enrichment culture showed the morphology and amount of Fe oxides deposited on Gallionella-like stalks (Figure 4C–E), which was likely related to bacterial growth phase. Apical cells were only observed in the stalked Gallionella-like culture by light microscopy, but not in microbial mat material or using SEM (Figure 4). Hallberg and Ferris (2004) also noted, using SEM, the absence of apical cells from Gallionella-like stalks in microbial mat samples recovered from underground drainage ditches. This is likely due to low apical cell densities and/or cell loss during sampling or sample treatment.

SEM analysis of unicellular Sideroxydans-like FeOB in liquid and semi-solid enrichment cultures showed cells at different degrees of mineral encrustation (Figure 5). The observation of cells devoid of Fe oxide encrustation, as well as Fe oxide deposition patterns, including globular nanometer-sized Fe oxides and crystallite Fe oxide crusts shown by SEM, and periplasmic Fe deposition shown by TEM, are similar to those observed with an anaerobic, nitrate-dependent FeOB, except we did not observe pole-specific localization of Fe biomineralization (Miot et al. 2009; Schadler et al. 2009). Despite the Fe oxide barrier, encrusted cells are likely to be metabolically active (Kappler et al. 2005b). Our findings provide the first direct evidence that FeOB may exhibit multiple Fe biomineralization patterns, similar to anaerobic FeOB. Further investigation is required to relate...
Fe biominerization patterns to the bacterial growth cycle of our laboratory enrichment cultures.

A growing consensus regarding Fe biominerization is that it could have played an important role for the deposition of large Fe accumulations in ancient oceans, including the Western Australian Hamersley group Banded-Iron-Formations (BIFs) during the Achaean era (Konhauser et al. 2002; Kappler et al. 2005a). Also, considering the generally high Fe and low nutrient content of Australian soils due to highly weathered profiles, we suggest FeOB have been of significance in the evolution of Australian regolith in both modern and historical contexts.

**Bacterial Fe Cycling Potential**

*In situ* Fe redox cycling rates and FeOB activities were not analyzed in this study. Based on relative abundance of FeOB in waters and mat material deposited on steel slides, we hypothesize that rapid Fe(II) oxidation dominates Fe biogeochemistry in surface waters including the mat-associated seep and shallow stream. Bacterial Fe(III) reduction was likely active and tightly linked to Fe(II) oxidation in the estuary-adjacent groundwater, where a large number of FeOB (10^5–10^7 cells mL^-1) and Fe(III)-reducing bacteria (FeRB, up to 10^8 cells mL^-1) were present (data not shown).

Biogenic Fe oxides are excellent electron acceptors which can be rapidly reduced through anaerobic Fe(III) respiration (Sobolev and Roden 2002; Emerson 2009). Hence, tightly linked bacterial Fe cycling has the potential to enhance overall Fe turnover rates with FeOB providing sufficient Fe(III) and organic carbon to sustain active FeRB populations (Sobolev and Roden 2002). Bacterial Fe cycling in subsurface aquatic environments may influence several environmentally significant biogeochemical processes, including organic matter mineralization, sulfate reduction and methanogenesis (Lovley and Phillips 1987; Chapelle and Lovley 1992; Canfield et al. 1993; Roden and Wetzel 1996; Teh et al. 2008). Stimulation of Fe redox cycling by FeOB and FeRB in Fe(II)-containing, low redox potential groundwater may lead to elevated Fe transformation and dissolution, increasing the risk of colloidal Fe mobilization and transport from the catchment into the estuarine system. We are currently examining the impact of Fe bacteria on these processes.

**CONCLUSIONS**

This study presents the first evidence for the ubiquity of FeOB in diverse surface and subsurface freshwater of a subtropical Australian coastal catchment. Results indicated that stalked *Gallionella*-, and sheathed *Leptothrix*-like FeOB were present in microbial mats associated with surface environments. In contrast, unicellular FeOB were widespread in surface and subsurface waters. We also present the first direct evidence for multiple patterns of Fe biominerization by unicellular FeOB. To our knowledge, this is the first culture-based survey of FeOB in an Australian context.

Our study demonstrates that laboratory cultivation using a variety of media in combination with molecular phylogenetic techniques are required to assess the ecology and diversity of FeOB in natural environments. The ubiquity of FeOB in Poona catchment aquatic habitats suggests bacterial Fe(II) oxidation is integral to the catchment Fe biogeochemistry. Further investigation of our laboratory enrichment cultures will help to characterize the ecology and physiology of FeOB in aquatic environments.

**REFERENCES**


Dyella koreensis sp. nov., a


