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Acid-tolerant microaerophilic Fe(II)-oxidizing bacteria promote Fe(III)-accumulation in a fen

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Summary

The ecological importance of Fe(II)-oxidizing bacteria (FeOB) at circumneutral pH is often masked in the presence of O_2 where rapid chemical oxidation of Fe(II) predominates. This study addresses the abundance, diversity and activity of microaerophilic FeOB in an acidic fen (pH ~5) located in northern Bavaria, Germany. Mean O_2 penetration depth reached 16 cm where the highest dissolved Fe(II) concentrations (up to 140 μM) were present in soil water. Acid-tolerant FeOB cultivated in gradient tubes were most abundant (10^6 cells g^{-1} peat) at the 10–20 cm depth interval. A stable enrichment culture was active at up to 29% O_2 saturation and Fe(III) accumulated 1.6 times faster than in abiotic controls. An acid-tolerant, microaerophilic isolate (strain CL21) was obtained which was closely related to the neutrophilic, lithoautotrophic FeOB *Sideroxydans lithotrophicus* strain LD-1. CL21 oxidized Fe(II) between pH 4 and 6.0, and produced nanoscale-goethites with a clearly lower mean coherence length (7 nm) perpendicular to the (110) plane than those formed abiotically (10 nm). Our results suggest that an acid-tolerant population of FeOB is thriving at redox interfaces formed by diffusion-limited O_2 transport in acidic peatlands. Furthermore, this well-adapted population is successfully competing with chemical oxidation and thereby playing an important role in the microbial iron cycle.

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Introduction

Aerobic Fe(II)-oxidizing bacteria (FeOB) in circumneutral environments are of special interest within the iron cycle due to their ability to compete with abiotic Fe(II)-oxidation in the presence of O_2 (Emerson and Moyer, 1997). Unlike highly acidic conditions (pH < 3), where Fe(II) persists despite the presence of O_2 (Stumm and Morgan, 1996; Cornell and Schwertmann, 2003), chemical oxidization of Fe(II) in the presence of molecular O_2 occurs rapidly under circumneutral conditions (Stumm and Morgan, 1996). Consequently, neutrophilic, aerobic FeOB often inhabit oxic-anoxic transition zones occurring in soils and sediments. These zones can form either as a result of O_2-consuming processes and slow subsequent delivery of O_2 from the water column (Brune *et al.*, 2000), adjacent to wetland plant roots due to O_2 release (Armstrong, 1964), or in groundwater seeps where anoxic water meets the atmosphere (Emerson and Moyer, 1997; Blöthe and Roden, 2009). So-called microaerophilic FeOB have generally been studied in or isolated from the rhizosphere of wetland plants (Emerson *et al.*, 1999; Neubauer *et al.*, 2002; 2007; Weiss *et al.*, 2007), aquatic sediments (Brune *et al.*, 2000; Sobolev and Roden, 2002; Wang *et al.*, 2009) or microbial mats at both freshwater (Emerson and Revsbech, 1994; James and Ferris, 2004; Rentz *et al.*, 2007; Blöthe and Roden, 2009) and marine sites (Edwards *et al.*, 2003; Emerson *et al.*, 2007). However, these studies did not address microbial Fe(II)-oxidation in environments more acidic (pH ~5.0) than circumneutral, where chemical oxidation is slower than under circumneutral conditions but relevant compared with acidic conditions (Meruane and Vargas, 2001). Given the slower oxidation rates in these slightly acidic environments, FeOB may contribute substantially to Fe(III) formation in acidic wetland habitats, such as the vast, nearly ubiquitous, peatlands in Eurasia and North America (Harriss *et al.*, 1993).

Minerotrophic peatlands (fens) vary in pH range (Mitsch and Gosselink, 2000) and microbial Fe(II)-oxidation in these habitats likely results from the substantial input of Fe(II) from groundwater flows (Küsel and Alewell, 2004; Todorova *et al.*, 2005). Fens provide a unique oxic-anoxic transition zone for Fe(II)-oxidation due to both the O_2 penetration in surface layers and the radial release of O_2 from plant roots. Chemical and microbial oxidation of

Fe(II) to Fe(III), followed by a precipitation of Fe(III) (hydr)oxides, is usually facilitated by these conditions, depending on the pH of the fen. We recently determined that accumulation of Fe(III) near the surface of an acidic fen (pH ~5) is enhanced when the water table is lowered during dry periods or after mixing with oxygenated rainwater (Reiche *et al*., 2009). This Fe(III) pool is rapidly depleted once O_2 is consumed (Reiche *et al*., 2009), and microbial Fe(III)-reduction can account for up to 70% of the anaerobic organic carbon mineralization in this fen (Küsel *et al*., 2008). This suggests that FeOB could play an important role by promoting a rapid microbial iron cycle in peatlands. In this study, we investigated the presence of acid-tolerant FeOB in a minerotrophic fen and evaluated the capacity of FeOB to compete with chemical oxidation. We also determined the abundance of acid-tolerant FeOB with respect to geochemical gradients and examined both the kinetics of Fe(II)-oxidation and the formation of Fe(III) (hydr)oxides under acidic conditions.

Results

Concentrations of dissolved Fe(II) and O_2 at the field site

Soil water concentrations of Fe(II) and the penetration depth of O_2 were measured in depth profiles obtained from May through August 2007. Between 10 and 15 cm below the surface, Fe(II) concentrations were the highest, reaching 140 μM in July and August 2007 (Fig. 1). The lowest Fe(II) concentrations were measured at 0–10 cm (< 50 μM) and 20–40 cm (< 80 μM) depths. In general, concentrations of nitrate were below the limits of detection (1.6 μM) and never exceeded 5 μM. The mean penetration depth of O_2 was 16 cm below the peat surface during this period (Fig. 1) with a maximum of 24.5 cm in June. These results were similar to those obtained in previous investigations at this sampling site (Paul *et al*., 2006; Küsel *et al*., 2008; Knorr *et al*., 2009; Reiche *et al*., 2009).

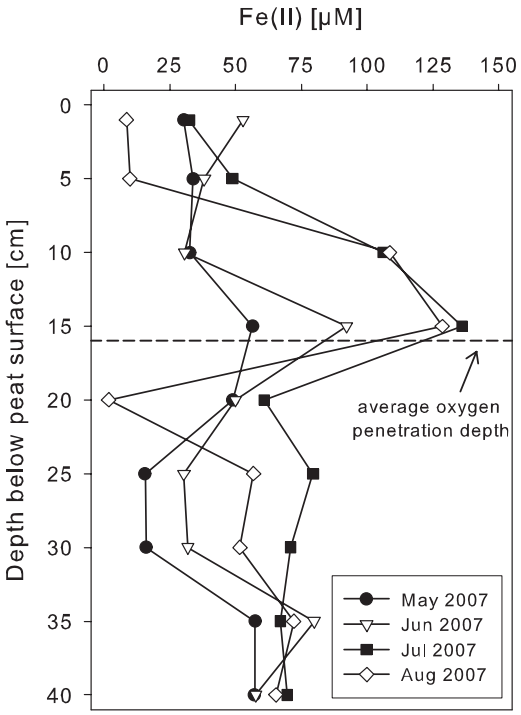

Fig. 1. Soil water profiles of Fe(II) over 0–40 cm from May to August 2007 obtained with Rhizon suction samplers. Greyish line is included to indicate the average O_2 penetration depth during the same time period.

Enumeration and enrichment of microaerophilic Fe(II)-oxidizers (FeOB)

Due to the absence of nitrate in the soil, we focused on enumerating microaerophilic FeOB capable of growth in gradient tubes with an initial pH of ~5.5. During incubation, the pH could decrease to a minimum of 4.4. FeOB abundance, as determined using an most probable number (MPN) technique, was approximately 1.2×10^5 cells g (wet weight peat)$^{-1}$ at 0–10 cm, 1.0×10^6 cells g (wet weight peat)$^{-1}$ at 10–20 cm and 2.0×10^3 cells g (wet weight peat)$^{-1}$ at 30–40 cm depths of a peat core obtained in December 2007. This was less than 0.02% of the total DAPI-stained microorganisms in the same depth segment determined at the sampling site in 2005 (Reiche *et al*., 2008). Different dilutions of the MPN enrichment cultures which exhibited both rapid and substantial Fe(III)-accumulation were selected for further enrichment and 16S rRNA gene-based community analysis. These were enrichments inoculated with 10^{-1} and 10^{-2} dilutions from the first depth segment (I: 0–10 cm), 10^{-3} dilution from the second depth segment (II: 10–20 cm) and 10^{-2} dilution from the forth segment (IV: 30–40 cm) of the gradient tube MPN series. Corresponding enrichments were named with SBIa, SBIb, SBII and SBIV according to their originating fen (SB: Schlöppnerbrunnen fen), their depth segment (I, II or IV) and with respect to their dilution (a for 10^{-1} and b for 10^{-2}). Enrichments from the 10–20 cm depth zone developed a distinct rust coloured band within 1 week of incubation. In contrast, enrichments obtained from depths above or below 10–20 cm, showed band development only after 3 weeks. Diffuse Fe(II)-oxidation above and below the rust coloured ring first appeared 8 weeks after inoculation. In sterile controls, the semisolid medium turned uniformly reddish orange after several days, suggesting chemical oxidation of Fe(II) as it was released from the FeS plug.

ARDRA, phylogenetic analysis and characterization of Fe(II)-oxidizer enrichments

A total of 151 16S rRNA gene clones were screened using amplified ribosomal DNA restriction analysis (ARDRA) and yielded 21 different phylotypes. Fourteen of these 21 phylotypes were present in SBIa, and eight and six phylotypes were present SBIb and SBII, respectively, while only four phylotypes were present in SBIV. Rarefaction curves of each clone library indicated a saturation of sampling (data not shown). The majority (43–76%) of sequenced 16S rRNA gene clones from the four clone libraries were most closely related to members of the *Betaproteobacteria*, specifically the *Sideroxydans lithotrophicus* strain ES-1, which is a known lithoautotrophic FeOB (Emerson and Moyer, 1997). Members of the *Alphaproteobacteria* comprised 17–38% of all clones and were dominated by species from the *Rhizobiales* and *Rhodospirillales*. Some of them are known as photosynthetic bacteria (Pfennig and Trüper, 1971) or nitrogen fixing organisms (Garg and Machanda, 2007). Phylotypes related to *Acidobacteria* were detected only in enrichment cultures SBIa and SBII (0–20 cm depth) and showed 95% similarity to Bacterium Ellin5239. *Acidobacteria* are omnipresent soil bacteria and also present in the fen investigated in this study (Schmalenberger *et al*., 2008). They are known for mediating Fe(III)-reduction in mine water-impacted environments (Pronk and Johnson, 1992; Blöthe *et al*., 2008; Coupland and Johnson, 2008).

Isolation of the acid-tolerant, microaerophilic Fe(II)-oxidizer strain CL21

We were only able to obtain an isolate from the fast growing SBII enrichment culture, called CL21, after four repeated dilution-to-extinction transfers. Each transfer was inoculated each time with the highest growth-positive culture from the previous dilution series. A cloning and sequencing approach confirmed that only one genotype was present. This culture formed a distinct rust coloured band in the gradient tubes and was unable to grow aerobically on plates specific for heterotrophs, suggesting the presence of a lithoautotrophic, microaerophilic FeOB. Cells had a curved, rod-shaped morphology with a length of 2–6 μm, with a cell diameter of 0.2 μm and CL21 cell surfaces were not encrusted with Fe(III) (hydr)oxides. Comparison of the 16S rRNA gene sequence of CL21 to known relatives revealed a sequence identity of 98.8% and 98.1% to that of *S. lithotrophicus* strain LD-1 (DQ386859), and to *S. lithotrophicus* strain ES-1 (DQ386264) respectively (Fig. 2). Both species belong to the beta-subdivision of the *Proteobacteria*. *Sideroxydans lithotrophicus* strain LD-1 was isolated from the rhizosphere of *Magnolia virginiana* in a bottomland hardwood forest (Weiss *et al*., 2007) and *S. lithotrophicus* strain ES-1 from an iron-mat formed in Fe(II)-containing groundwater (Emerson and Moyer, 1997). Both species have been described as microaerophilic FeOB.

Oxygen microprofiles

Gradient tubes inoculated with the SBII enrichment had steeper O_2 gradients compared with sterile gradient tubes (Fig. 3). In both, inoculated gradient tubes and sterile controls, the depth of oxygen penetration decreased over time and remained stable after 9 and 5 days respectively. In inoculated tubes a distinct rust coloured band with an O_2 concentration of 29% on top was formed. The overlayer of the sterile controls turned uniformly reddish orange and became darker in colour during the incubation period.

The O_2 concentration on top of the rust coloured band of isolate CL21 was approximately 17.5% 14 days after inoculation and was depleted to 0% within the band (data not shown). In some gradient tubes inoculated with CL21, single colonies grew above the band at O_2 concentrations of approximately 30%.

Fe(II)/Fe(III) and pH profiles

Profiles of Fe(II) and Fe(III) were measured in inoculated and sterile gradient tubes at 5 mm vertical increments during 12 days of incubation of the SBII enrichment culture. Fe(II) concentrations in the first 10 mm of the SBII tubes were approximately 0.2 mM and increased from 0.9 mM at 15 mm depth to 3.4 mM Fe(II) adjacent to the FeS plug at a depth of 37 mm. Fe(III) accumulation was highest between 10 and 15 mm and increased to a its maximum concentration of 6.2 mM Fe(III) within the first 8 days of incubation (Fig. 4). Fe(II) concentrations in sterile controls increased with increasing depth from 1.3 to 3.6 mM, while Fe(III) concentrations increased from approximately 1 to 2.3 mM at the bottom of the overlayer. The zone of highest Fe(III) accumulation was between 25 and 35 mm below the surface (Fig. 4).

Within the first 12 days of incubation, Fe(III) accumulation increased linearly to 1.75 and 1.79 mM Fe(III) in the overlayer of gradient tubes inoculated with either the enrichment SBII or isolate CL21 at a rate of 0.15 and 0.17 mM Fe(III) day^{-1} (Fig. 5). Fe(III) concentrations measured in the sterile controls also increased during the same period to 1.14 mM Fe(III) at a rate of 0.1 mM Fe(III) day^{-1}. Therefore, the rate of Fe(III) accumulation was 1.5–1.6 times higher in the inoculated tubes. In contrast to sterile controls, rates of Fe(III) accumulation decreased during the following 14 days in the inoculated tubes. After 26 days of incubation, Fe(III) concentrations were similar in inoculated and sterile gradient tubes at 2.6–2.8 mM Fe(III) respectively (Fig. 5).

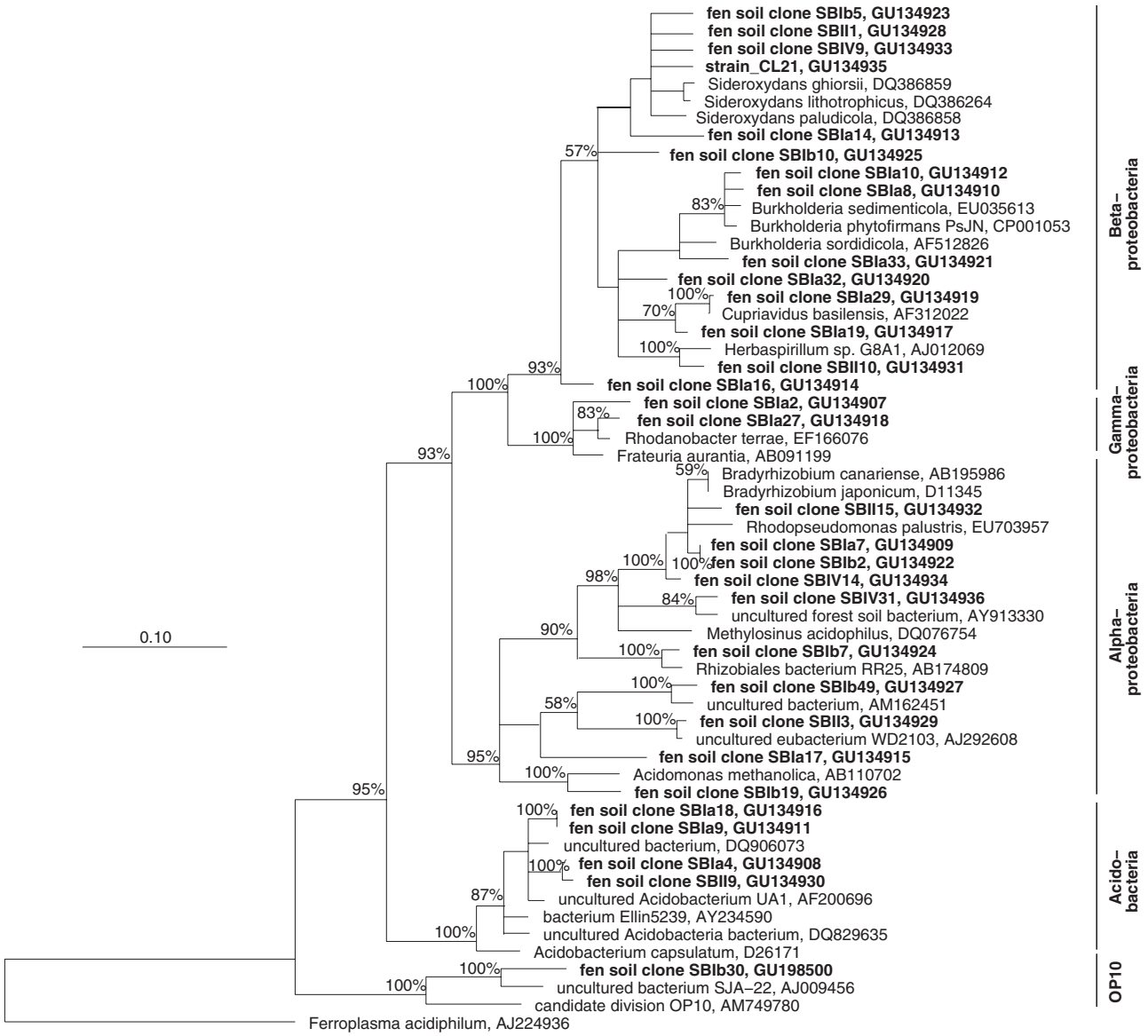

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences (indicated by boldface type) derived from gradient tube enrichments from peat zones 0–10 cm (I), 10–20 cm (II) and 30–40 cm (IV) and isolate CL21. The tree represents a consensus of the phylogeny determined using neighbour-joining, maximum-parsimony and maximum-likelihood methods. Parsimony bootstrap values (1000 data resamplings) ≥ 50% are given at branch points. Scale bar represents 10 nucleotide substitutions per 100 positions. The outgroup *Ferroplasma acidiphilium* (AJ224936) was used for calculations of the bacterial tree.

The pH measured in the middle of the tubes, above and below the distinct rust coloured band formed by CL 21, decreased from pH 5.5 in unbuffered medium to ~5.0 after 12 days of incubation, and then dropped to its final value of 4.4 over the next 10 days. No further decrease in pH values was measured after 29 days of incubation. In sterile controls, the pH decreased only to 5.2 over the same incubation period. Isolate CL21 has the ability to oxidize Fe(II) within the pH range of 4.0–6.0. A distinct rust coloured band was formed at a pH of 6.0 but not at a pH of 6.2 and 6.5 even after a couple of weeks. The lower pH value (pH 4.0) was measured in active cultures of CL21. The top layer did not solidify at pH lower than 4; thus, we cannot rule that CL21 might be active also at lower pH. Maximum rates of Fe(II)-oxidation were observed at 21°C (Table 1 and data not shown). Abiotic oxidation rates of Fe(II) in gradient tubes incubated at different temperatures were low at the initial pH of 4.3 or 5 and at low temperatures (Table 1).

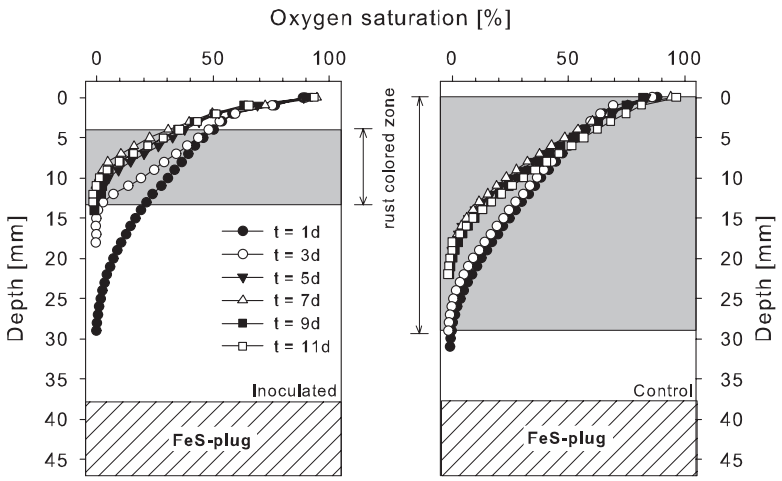

Fig. 3. Oxygen microelectrode profiles in inoculated (left: enrichment culture SBII) and sterile (right) gradient tubes over an incubation period of 13 days. Greyish boxes are included to indicate the area of microbial and chemical Fe(II)-oxidation relative to the oxygen concentrations.

Characterization of the Fe(III) solid phase

In both inoculated (isolate CL21 and enrichment SBII) and sterile gradient tubes after 7 weeks of incubation, X-ray diffraction (XRD) identified goethite (alpha-FeOOH) as the main solid product of Fe(II)-oxidation. A peak at d = d-spacing [nm] = 0.269 appeared only in the tube inoculated with SBII and is consistent with the main peak of hematite (Fe_2O_3). In all samples, one broad peak with a day-spacing of 0.58–0.60 nm could not be identified, but is not in accordance with typical Fe oxides or green rust (Cornell and Schwertmann, 2003). The low signal to noise ratio and the large width of the observed goethite XRD peaks point to a small crystal size and/or a poor crystal order. Furthermore, goethites formed at high ionic strength and low temperatures, as in our experiments, are known to often develop a multidomainic structure (Cornell and Schwertmann, 2003). When neglecting the effect of lattice imperfections, this domain size can be estimated from the width of the XRD peaks (Scherrer, 1918). Using the Scherrer equation, we found a mean domain size of 10 nm for the sterile control, whereas the goethites of both inoculated tubes consisted of 7 nm large domains.

Energy-dispersive X-ray spectroscopy (EDX) spectra confirms the XRD results by showing signals for O and Fe. Besides the goethites, we observed some Fe-free needle-shaped and plate-like crystals, with an EDX signal of S, O, Na, Mg and Cl (data not shown). We assume that these are salts crystals precipitated from the mineral salts medium. In general, goethite appeared associated to the net-like agar matrix.

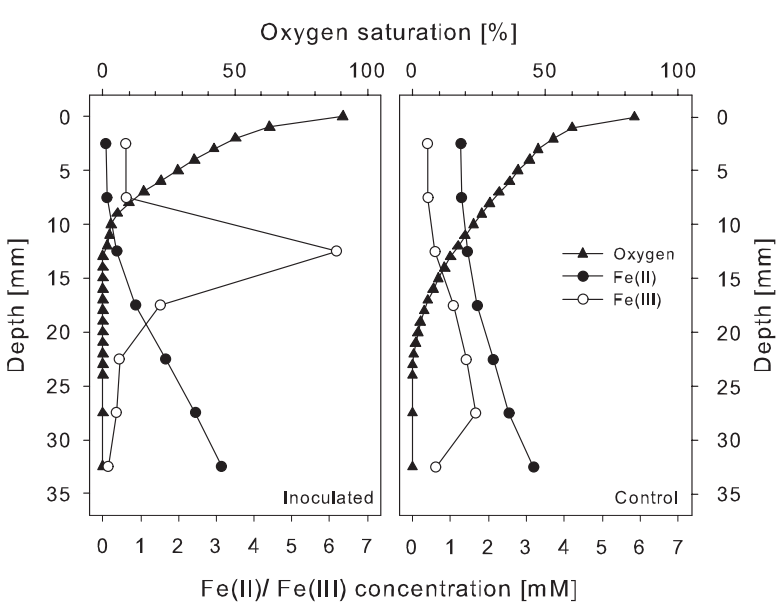

Fig. 4. Oxygen and Fe(II)/Fe(III) profiles measured in gradient tubes inoculated with enrichment culture SBII (left) 8 days after inoculation. A sterile tube was used as control (right).

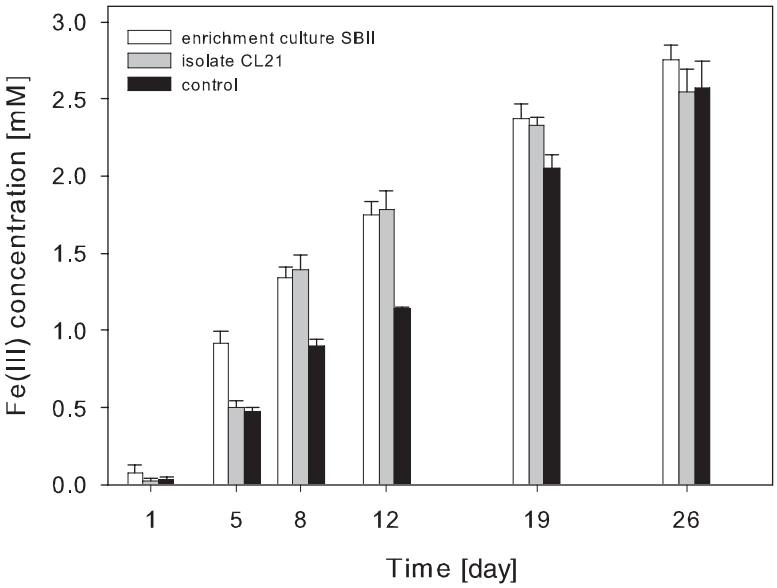

Fig. 5. Fe(III) accumulation over time determined in the whole overlayer of gradient tubes inoculated with enrichment SBII and isolate CL21, both obtained from the 10–20 cm zone of an acidic fen. Sterile tubes were used as a control.

Discussion

Abundance of microaerophilic Fe(II)-oxidizers in oxic/anoxic gradients

The oxic/anoxic transition zone with the highest Fe(II) concentrations within a minerotrophic fen in northern Bavaria, Germany contained high numbers of acid-tolerant FeOB [up to 10^6 cells g (wet weight peat)$^{-1}$]. In general, the highest potential for microbial Fe(II)-oxidation in this fen is most likely within the upper 20 cm of the peat, due to the presence of an oxic to suboxic transition zone. This zone of potential Fe(II)-oxidation can be enlarged temporarily to 40 cm due to a low water table during dry seasons (Reiche *et al*., 2009), which would explain the presence of microaerophilic FeOB in depths below 30 cm [10^3 cells g (wet weight peat)$^{-1}$]. MPN numbers of FeOB present in the peat are similar to MPNs of neutrophilic FeOB from Fe mats in a groundwater seep-fed wetland (10^6 cells ml mat^{-1}) at Fe(II) concentrations of approximately 200 μM (Druschel *et al*., 2008). In those mats, voltammetric profile measurements indicate that the mat becomes suboxic/anoxic within 2 mm of the surface, and the overlapping zone of available O_2 and Fe(II) is less than 1 mm. Another study found that, in the rhizosphere of *Typha* spp., numbers of aerobic, lithotrophic FeOB are in a similar range to what we found [10^5–10^6 cells g (root)$^{-1}$] (Weiss *et al*., 2003), but the zone of microbial Fe(II)-oxidation still appears to be restricted to several millimetres around the roots (Mendelssohn *et al*., 1995). This suggests that the oxic/anoxic transition zone present in mats and the rhizosphere is very thin compared with the oxygenated surface layers in peatlands where microaerophilic FeOB might be active. Anaerobic, nitrate-dependent and anaerobic phototrophic FeOB (Widdel *et al*., 1993; Straub and Buchholz-Cleven, 1998; Kappler and Newman, 2004; Weber *et al*., 2006) should only play a minor role in this fen due to the low availability of nitrate (Verhoeven, 1986; Vepraskas and Faulkner, 2001; Küsel *et al*., 2008; Reiche *et al*., 2009), and the low light penetration in soils of about 200 μm (Ciani *et al*., 2005).

Diversity of Fe(II)-oxidizing microorganisms

A comprehensive phylogenetic analysis of cultivated FeOB 16S rRNA genes revealed a diverse community that was dominated by members of the *Betaproteobacte-*

Table 1. Fe(III) accumulation rates (μM day^{-1}) obtained from opposing gradient tube incubations.

	Microbial Fe(II)-oxidation[a]	Chemical Fe(II)-oxidation[b]			
Incubation temperature	pH 4.6	pH 4.3	pH 5.0	pH 6.7	pH 7.3
4°C	30.1	7.3	17.2	44.6	53.5
15°C	32.9	9.4	19.6	59.5	59.2
21°C	44.0	17.5	41.5	64.5	71.3
30°C	33.9	33.0	57.6	73.1	81.7

a. Microbial Fe(II)-oxidation was determined after inoculation with isolate CL21 after 6 weeks of incubation.
b. Chemical Fe(II)-oxidation was obtained in sterile gradient tubes after 3 weeks of incubation.

ria. The data support the observation that lithotrophic FeOB primarily associated with freshwater environments are members of the *Betaproteobacteria* (Weiss *et al*., 2007), whereas marine FeOB typically belong to the novel class *Zetaproteobacteria* (Emerson *et al*., 2007). Since the majority of sequenced 16S rRNA gene clones from the enrichment culture SBII were most closely related to *Sideroxydans* spp., the activity of these organisms may be responsible for the similarity in Fe(II)-oxidation rates with isolate CL21. Surprisingly, 29% of the SBII clones obtained from the 10–20 cm depth zone were closely related to the purple non-sulfur phototrophic organism *Rhodopseudomonas palustris*. Under anoxic conditions, *R. palustris* strain TIE-1 can grow photoautotrophically with Fe(II), hydrogen or thiosulfate as electron donors and photoheterotrophically with a variety of organic carbon sources (Jiao *et al*., 2005). In SBIa, 19% of obtained clones were related to *Frateuria aurantia*, a moderately acidophilic Fe(II)-oxidizer (Hallberg and Johnson, 2005) within the *Gammaproteobacteria*. In enrichment cultures SBIa and SBII, clones with high sequence similarity to members of the *Acidobacteria* were found. The *Frateuria*-like strain WJ2 and members of the *Acidobacteria* have been shown to reduce Fe(III) (Pronk and Johnson, 1992; Hallberg and Johnson, 2005; Blöthe *et al*., 2008; Coupland and Johnson, 2008). The presence of potential Fe(III)-reducing organisms in the FeOB enrichments suggests both a microscale Fe-cycling in the opposing gradient tubes and at oxic-anoxic interface in the fen. A microbial microscale cycling of Fe was also shown at distinct sand-water interfaces (Roden *et al*., 2004).

Sideroxydans strain CL21

A physiologically unique, acid-tolerant *Sideroxydans* isolate, strain CL21, was obtained from a moderately acidic minerotrophic fen that could oxidize Fe(II) at a low pH range. Strain CL21 could grow only chemolithoautotrophically, as heterotrophic growth was not observed by CL21. However, CL21 grew in a pH range of 4.0–6.0, but not at higher pHs, which differs from the optimal pH range of *Sideroxydans* LD-1 (4.5–7.0) or ES-1 (5.5–7.5) (Emerson and Moyer, 1997; Weiss *et al*., 2007). Since CL21 also had a lower pH optimum (4.5–5.0) than the others, CL21 can be classified as the first acid-tolerant *Sideroxydans* species. CL21 could also tolerate lower temperatures (3.5°C) than ES-1 or LD-1 (10°C and 12°C respectively) suggesting that this strain is well adapted to the cold, acidic conditions commonly found in this fen. This finding challenges again the concept of species differentiation based on sequence similarity with 16S rRNA genes. Cell morphology of CL21 was also different from the strains closely related by sequence identity. The diameter of CL21 cells (0.2 μm) were less than half the diameter of LD-1 (0.74 μm) but similar to that of ES-1 (0.32 μm). Scanning electron microscopy (SEM) pictures also showed that CL21 have flagella at the polar side of the cell. Cells of our isolate, CL21, were associated with particulate Fe(III) (hydr)oxides, but were not encrusted, similarly to ES-1 (Emerson and Moyer, 1997).

Competition between chemical and microbial Fe(II)-oxidation

The activity and biogeochemical role of FeOB in neutral and extremely acidic environments is well established (Baker and Banfield, 2003; Emerson and Weiss, 2004). Neutrophilic FeOB must compete with rapid chemical oxidation of Fe(II) in the presence of oxygen. Consequently, these FeOB thrive only in niches with low oxygen concentrations where chemical oxidation Fe(II) by oxygen is slow (Emerson, 2000). In contrast, under highly acidic conditions (pH < 3) no chemical oxidation of Fe(II) occurs, even under oxygen saturated concentrations. Chemical oxidation starts at pH values above 4.2 with maximum rates achieved at pH values above 5.5 (Meruane and Vargas, 2001). The abiotic oxidation rates of Fe(II) measured in our gradient tubes, incubated at 4 or 15°C, were not only low at the initial pH of 5, but were even lower at pH 4.3 (Table 1). Thus, the acid-tolerant FeOB present in this fen may be able to compete successfully with chemical oxidation of Fe(II) at *in situ* pH and temperatures, as the pH of the peat soil varied between 4.0 and 5.2 and the average peat soil temperature at 20 cm depth was approximately 6.5°C (G. Lischeid, pers. comm.). High temperatures (21 and 30°C), which favour chemical Fe(II)-oxidation (Table 1), are unlikely in this fen, where the maximum temperature reached only 16°C over the 2 years of study.

At 21°C and an initial pH of about 5.5, FeOB increased Fe(II)-oxidation rates by 1.6 times relative to chemical oxidation in sterile controls. Subtracting the biotic rate from potential background chemical oxidation, the minimum reaction attributed to the biotic component ranged from 32% to 35% within the first 12 days of incubation (Fig. 5). This range is likely strongly underestimated. First, the amount of Fe(III) formed during chemical oxidation occurred over a large reaction zone of more than 1 cm as compared with the restricted zone of Fe(II)-oxidation in the inoculated gradient tubes where the majority of Fe(III) precipitated in a small ring. Second, simultaneous microbial and chemical oxidation might have not even occurred in the ring. Finally, the decline in pH during Fe(III) hydrolysis and precipitation associated with microbial Fe(II)-oxidation would have negatively affected chemical oxidation in the opposing gradient cultures.

At pH 6.2, *S. lithotrophicus* strain ES-1 increases the rate of Fe(III) accumulation 1.1 to 1.7 times with increasing O_2 concentrations in electrochemical cells from 9 to 50 μM (equals 3.3–18.2% saturation) (Druschel *et al*., 2008). Biotic oxidation makes up 15–43% of the total rate. Also, batch cultures of *Sideroxydans paludicola* strain BrT grown in bioreactors at circumneutral pH indicate that strain BrT accounts for at least 18–53% of the total Fe(II)-oxidation (Neubauer *et al*., 2002). Results obtained from flow chamber experiments suggest that 50–80% of the Fe(II)-oxidation is mediated by microbes of a mat community at neutral pH (Emerson and Revsbech, 1994). However, the study of Sobolev and Roden (2001) shows that even though rates of microbial and chemical Fe(II)-oxidation are similar in neutral-pH opposing gradient cultures, FeOB are likely to account for nearly all ($\geq$ 90%) of the Fe(II)-oxidation. Hence, the biotic catalysis might be also responsible for the vast majority of Fe(II)-oxidation in the above-mentioned experimental studies. As a consequence, the used methodologies and the basic strategy to use the ratio of biotic vs. abiotic rates to infer the likely relative importance of microbial catalysis reflect limitations. Alternatively, possible new gene expression-based approaches might be applied to address the issue as our understanding of the genetics and biochemistry of FeOB advances.

Isolate CL21 was active at O_2 concentrations up to 17.5%, which is in the range of that observed for ES-1 (15–50 μM which is equal to 5.5–18.2%) (Druschel *et al*., 2008). Single colonies in the semisolid agar were active even up to 30% O_2 saturation at pH 4.8. Druschel and colleagues (2008) suggested that abiotic Fe(II)-oxidation is favoured above 18.3% O_2 at a pH of about 6.2, which then limits the niche in which circumneutral FeOB thrive. Other results obtained with neutrophilic FeOB support this upper O_2 limit of up to 20% saturation (Sobolev and Roden, 2001). Therefore, more acidic conditions apparently increase the O_2 window for microbial Fe(II)-oxidation from near 20% up to 30% O_2 saturation. Thus, the ability of acid-tolerant FeOB to compete more successfully with abiotic Fe(II)-oxidation at higher dissolved O_2 concentrations than FeOB under neutral pH conditions might also be responsible for the relatively wide depth distribution of these organisms in the fen.

Formation of goethite by microbial and chemical oxidation

Nanoscaled-goethite was found as the main product of chemical (sterile control) and microbial (inoculated tubes) Fe(II)-oxidation in our gradient incubations. Mineral products of microbial Fe(II)-oxidation have been shown to be pH dependent, as below pH 7.0, goethite (alpha-FeOOH) forms, and above pH 7.2, magnetite (Fe_3O_4) forms (Jiao *et al*., 2005).

The goethites of the sterile tubes showed a clearly larger domain size (10 nm) than the goethites produced in the inoculated tubes (7 nm). Crystal size depends on growth conditions such as the degree of oversaturation, the dominating transport processes in solution and the presence of foreign ions or molecules. Accordingly, the smaller goethites in the inoculated tubes may be explained by the higher concentration of Fe(III) ions in the distinct ring zone or by the higher concentration of extracellular polymeric substances produced by the organisms. XRD patterns obtained from Fe(III)-rich surface peat from the fen site yielded no positive mineral identification for Fe (hydr)oxides (data not shown), indicating the need for Fe specific or spatially resolved analysis methods in soil samples.

Potential for microbial cycling of iron in an acidic fen

In minerotrophic, groundwater-influenced peatlands mineral element cycles, like the cycling of iron, are more common than often acknowledged. A lot of studies showed a significant presence of iron in such systems (e.g. Hajek and Hekera, 2004; Dettling *et al*., 2006; Boomer & Bedford, 2008; Deppe *et al*., 2010) or even revealed microbial Fe(III) reduction to play a major role in peat mineralization processes (Todorova *et al*., 2005; Metje & Frenzel, 2007; Reiche *et al*., 2008; Jerman *et al*., 2009). Similar to microbial Fe(III) reduction, the biotic Fe(II)-oxidation in peatlands is poorly understood. In the present study we could show that microbial Fe(II)-oxidation enhanced Fe(III) accumulation in gradient tubes. The microbially produced Fe(III) (hydr)oxides were more fine grained compared with the chemical oxidation products. It was shown that Fe(III)-reducing microorganisms profit from high specific surface areas of the formed Fe(III) minerals (Roden, 2003). In previous investigations we could show that a high microbial available Fe(III) pool accumulated in surface peat layers of this fen (Reiche *et al*., 2008) which might be mainly provided by the microbial oxidation of Fe(II). These results indicate a potential coupling of microbial Fe(II)-oxidation and Fe(III) reduction at redox interfaces in iron-rich fens, thereby promoting rapid microscale cycling of Fe.

Conclusion

Our data highlight the importance of acid-tolerant FeOB in an acidic fen which contributed substantially to the oxidation and accumulation of Fe(III) under low temperature and pH conditions of about 5. The extent of the subsurface potentially available for microbially mediated Fe(II)-oxidation in peatlands is much larger (cm scale) than in mat or rhizosphere systems as it is not restricted to only a few millimetres.

Experimental procedures

Field site and sampling

Peat samples were obtained from a minerotrophic, acidic fen (Schlöppnerbrunnen, fen area: 0.8 hectare) in the Lehstenbach catchment area located in the northern Fichtelgebirge region in east-central Germany (50°7′54″N, 11°52′51″E at an altitude of 700 m above sea level) as previously described (Reiche *et al*., 2008; 2009). Peat in the first 10 cm is enriched with high amounts [approximately 200 μmol Fe g (dry wt peat)$^{-1}$] of microbially available Fe(III) (Reiche *et al*., 2008; 2009).

Peat samples from 0–40 cm depth were obtained using an 8 cm diameter peat corer in May and December 2007, and July 2008 from the middle of the fen (plot C2; Reiche *et al*., 2009). Fresh plant litter was removed from the top and cores were separated into 10 cm depth segments (I: 0–10 cm, II: 10–20 cm, IV: 30–40 cm). Peat samples were then transported to the laboratory in airtight plastic bags at 4°C and processed the same day.

The penetration depth of O_2 was determined in August and October 2006 and from May through August 2007 using FeS-redox-probes as described in Reiche and colleagues (2008; 2009). A colour change of the probes from black to brown indicated the presence of O_2 due to the oxidation of FeS to Fe(III) (hydr)oxides. Soil water was obtained during the same period close to the 8 cm core sampling site from previously installed Rhizon suction samplers (Reiche *et al*., 2008; 2009).

Cultivation and isolation of microaerophilic Fe(II)-oxidizers

For enrichment, culturing and the enumeration of microaerophilic FeOB, a modified gradient tube technique (Emerson and Moyer, 1997) was used. Opposing gradients of O_2 and Fe(II) were established in 10 cm by 1.2 cm screw-cap glass tubes. The tubes had a 750 μl plug of FeS [FeS mixed 1:1 with modified Wolfe's mineral medium (MWMM) (Hanert, 1992) and 3% (wt/vol) agarose] overlaid with 6 ml semisolid mineral salts – medium [MWMM, 0.2% (wt/vol)] agarose, 9 μl vitamin B solution (Drake, 1994) and a headspace of air. The total height of the overlayer medium was 37 mm. While still molten, the overlayer was bubbled with filter-sterilized CO_2 for 5 s and tubes were capped. The final pH in the unbuffered medium was around 5.5. Tubes were opened under air, 20 μl of inoculum were expelled from the FeS plug to the top as a pipette tip was withdrawn (Hanert, 1992) and incubated at 21°C in the dark. Changes in pH during Fe(II)-oxidation were determined with a pH probe (diameter of tip: 5 mm, SenTix 97T, WTW, Germany). To test the pH range of chemical Fe(II)-oxidation, the semisolid mineral salts – medium was buffered with 10 mM HEPES, and the pH was adjusted with hydrochloric acid or sodium hydroxide to reach pH values between 4.0 and 7.3. To prevent hydrolysis at lower pH values, 0.4% gellan gum (0.4 g $MgSO_4$*7 H_2O per gram gellan gum) instead of agarose was used for solidification of the top layer of the medium. These tubes were incubated in the dark at temperatures from 4.0°C to 30.0°C. The upper pH limit for CL21 was repeatedly tested in bicarbonate-buffered gradient tubes at pH values of 6.0, 6.2 and 6.5. In general, active cultures were transferred to new gradient tubes within 8 weeks after inoculation. The dilution-to-extinction cultivation method was then used to obtain isolates. To test for heterotrophic growth under oxic conditions, cultures were streaked on solid plates containing per 1 L: 3 g yeast extract, 1.5 g peptone and 1.5 g tryptone, and 15 g agar.

Enumeration of microaerophilic Fe(II)-oxidizers

Numbers of culturable FeOB were determined by the MPN technique with three replicates per dilution in anoxic MWMM and incubated for 6 weeks in gradient tubes as described above. Tubes were considered positive based on the formation of a distinct rust coloured band compared with the diffuse Fe(II)-oxidation in uninoculated controls. MPN values were then calculated from standard MPN tables within 95% certainty (de Man, 1975; 1977).

Activity of microaerophilic Fe(II)-oxidizers

To investigate the activity of enriched Fe(II)-oxidizers and to distinguish between chemical and microbial Fe(II)-oxidation, O_2, pH, Fe(II) and Fe(III) profiles were determined in the gradient tubes over time. Oxygen profiles were measured at increments of 1 mm with a microelectrode (diameter of tip: 100 μm, Unisense, Denmark) every 48–72 h during the minimum 14 day incubation period. pH profiles were measured weekly, over an incubation period of 1 month, 15 mm above, within, and 15 mm below the distinct, rust coloured band with a pH probe (diameter of tip: 5 mm, SenTix 97T, WTW, Germany). Fe(II) and Fe(III) profiles were measured after HCl extraction (0.5 M) of 5 mm increments of the semisolid medium every 2 or 3 days during the first 12 days of incubation The accumulation of Fe(III) was determined 6 times within an incubation period of 26 days by HCL extraction of the whole semisolid medium (6 ml overlayer added to 114 ml 0.5 M HCl). Measurements were performed always in three separate replicate gradient tubes, which were sacrificed. Sterile tubes acted as a control.

Analytical techniques

Fe(II) was measured after centrifugation of HCl extracts following the phenanthroline method after Tamura and colleagues (1974). HCl extractable Fe(III) was calculated after the addition of ascorbic acid (0.6% final concentration) from the increase in Fe(II) concentration. Nitrate was analysed by ion chromatography.

The mineral products of Fe(II)-oxidation were measured by SEM according to Schädler and colleagues (2008) described below and by powder XRD analysis. XRD patterns were obtained with a Seifert-FPM XRD 7 goniometer using Cu Kα radiation at 40 kV and 30 mA, step-scanning from 5 to 80 °2Θ with increments of 0.02 °2Θ and a counting time of 20 s per step. The diameter of coherent scattering domains $D_{Scherrer}$ was calculated by applying the Scherrer equation to the (110) peak of goethite (Scherrer, 1918): $D_{Scherrer} = 0.9\ \lambda/FWHM_{true} \cos\theta$, where λ is the wavelength, $FWHM_{true}$ the true FWHM in radians and θ the diffraction angle. The true width of the XRD

peak at its half maximum was determined by $FWHM_{true} = (FWHM_{sample}^2 - FWHM_{std}^2)^{0.5}$, where $FWHM_{sample}$ is the FWHM of the sample profile and $FWHM_{std}$ is the FWHM of the same peak from a standard sample (Klug and Alexander, 1974). A well crystalline synthetic goethite served as the standard in this study. The diffraction angle and FWHM values were obtained by profile fitting with XFIT (http://www.ccp14.ac.uk/tutorial/xfit-95/xfit.htm) using a linear background and Pseudo-Voigt functions.

Electron microscopy

The morphology of isolate CL21 was determined using SEM. A sub sample of the culture was collected on a lysine-coated cover slide by sedimentation followed by a standard SEM preparation procedure (Bozzola, 2007). All samples were examined with a FE-SEM LEO-1530 Gemini (Carl Zeiss NTS, Germany) at magnifications of 15 000x to 20 000x. Crystal-like structures were additionally analysed using EDX utilizing a Quantax 200 XFlash detector (Bruker AXS Microanalysis, Germany) at a LEO-1450 (Carl Zeiss NTS, Germany).

16S rRNA gene sequencing and phylogenetic analysis

The distinct rust coloured band of the enrichment cultures was transferred to reaction tubes and cell material was concentrated by centrifugation (5 min at 10 000 *g*). Genomic DNA was extracted from the pellet using the PowerSoil DNA Isolation Kit according to the manufacturer's instructions (MO BIO Laboratories, USA). DNA extracts were PCR amplified with the *Bacteria* domain-specific 16S rRNA gene primers fd1 and rp2 (Weisburg *et al*., 1991). Thermocycling was performed according standard procedure with 30 cycles and an annealing temperature of 56°C. Purified PCR products were cloned into the TOPO TA cloning vector pCR 2.1 (Invitrogen, USA) or pGEM-T Vector System (Promega, USA) according to the manufacturer's instructions. Cloned inserts were PCR amplified using vector-specific primers (M13F/M13R) then digested with restriction enzymes MspI and BshFI (0.25 U μl^{-1}, Fermentas, Germany). Clones were grouped into phylotypes according ARDRA band patterns and 1–2 representative clones per phylotype were sequenced bidirectionally (Macrogen, South Korea). Sequences were analysed using the ARB software package. Trees were constructed using parsimony, maximum likelihood and neighbour-joining methods and 1000 bootstrap replicates were performed (Felsenstein, 1985). The sampling efficiency in clone libraries was assessed using Analytical Rarefaction version 1.3 software (Holland, 2003).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences have been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov) under accession numbers GU134907 to GU134936, and GU198500 (see Fig. 2).

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