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Growth and Population Dynamics of the Anaerobic Fe(II)-Oxidizing and Nitrate-Reducing Enrichment Culture KS

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ABSTRACT Most isolated nitrate-reducing Fe(II)-oxidizing microorganisms are mixotrophic, meaning that Fe(II) is chemically oxidized by nitrite that forms during heterotrophic denitrification, and it is debated to which extent Fe(II) is enzymatically oxidized. One exception is the chemolithoautotrophic enrichment culture KS, a consortium consisting of a dominant Fe(II) oxidizer, *Gallionellaceae* sp., and less abundant heterotrophic strains (e.g., *Bradyrhizobium* sp., *Nocardioides* sp.). Currently, this is the only nitrate-reducing Fe(II)-oxidizing culture for which autotrophic growth has been demonstrated convincingly for many transfers over more than 2 decades. We used 16S rRNA gene amplicon sequencing and physiological growth experiments to analyze the community composition and dynamics of culture KS with various electron donors and acceptors. Under autotrophic conditions, an operational taxonomic unit (OTU) related to known microaerophilic Fe(II) oxidizers within the family *Gallionellaceae* dominated culture KS. With acetate as an electron donor, most 16S rRNA gene sequences were affiliated with *Bradyrhizobium* sp. *Gallionellaceae* sp. not only was able to oxidize Fe(II) under autotrophic and mixotrophic conditions but also survived over several transfers of the culture on only acetate, although it then lost the ability to oxidize Fe(II). *Bradyrhizobium* spp. became and remained dominant when culture KS was cultivated for only one transfer under heterotrophic conditions, even when conditions were reverted back to autotrophic in the next transfer. This study showed a dynamic microbial community in culture KS that responded to changing substrate conditions, opening up questions regarding carbon cross-feeding, metabolic flexibility of the individual strains in KS, and the mechanism of Fe(II) oxidation by a microaerophile in the absence of O_2.

IMPORTANCE Nitrate-reducing Fe(II)-oxidizing microorganisms are present in aquifers, soils, and marine and freshwater sediments. Most nitrate-reducing Fe(II) oxidizers known are mixotrophic, meaning that they need organic carbon to continuously oxidize Fe(II) and grow. In these microbes, Fe(II) was suggested to be chemically oxidized by nitrite that forms during heterotrophic denitrification, and it remains unclear whether or to what extent Fe(II) is enzymatically oxidized. In contrast, the enrichment culture KS was shown to oxidize Fe(II) autotrophically coupled to nitrate reduction. This culture contains the designated Fe(II) oxidizer *Gallionellaceae* sp. and several heterotrophic strains (e.g., *Bradyrhizobium* sp.). We showed that culture KS is able to metabolize Fe(II) and a variety of organic substrates and is able to adapt to dynamic environmental conditions. When the community composition changed and *Bradyrhizobium* became the dominant community member, Fe(II) was still oxidized by *Gallionellaceae* sp., even when culture KS was cultivated with acetate/nitrate [Fe(II) free] before being switched back to Fe(II)/nitrate.

KEYWORDS autotrophic nitrate-dependent Fe(II) oxidation, *Gallionellaceae*, CARD-FISH

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As an essential component of life, iron (Fe) is involved in numerous physiological reactions and is also a widely distributed redox-active element in nature that plays an important role in geochemical processes. Reduced ferrous Fe [Fe(II)] and oxidized ferric Fe [Fe(III)] are the two most important redox states of Fe in the environment and are part of the biogeochemical Fe cycle. Several biotic and abiotic processes are involved in the reduction of Fe(III) as well as in the oxidation of Fe(II), connecting Fe with other element cycles, such as the carbon (C), sulfur (S), and nitrogen (N) cycles (for examples, see references 1–5, and 6; for reviews, see references 7 and 8).

Under anoxic conditions, Fe(II) can be oxidized abiotically by reactive N species, such as nitrite and nitric oxide, which are produced during denitrification (9–14). There are also several microorganisms described from freshwater, brackish water, and marine sediments as well as soils that can use nitrate as an electron acceptor and Fe(II) as an electron donor (1, 3, 4, 15–19). However, more than 20 years after the first discovery of nitrate-reducing Fe(II) oxidizers (NRFO) by Straub et al. (1), the molecular mechanism(s) of Fe(II) oxidation is still unclear. Although putative extracellular electron transfer mechanisms (e.g., by outer membrane cytochromes or by a "porin-cytochrome *c* protein complex"), which are potentially involved in Fe(II) oxidation, are postulated for NRFO (20), it is not known whether or to what extent nitrate-dependent Fe(II) oxidation is enzymatically catalyzed or whether it is mainly a side reaction of reactive N species produced as by-products during denitrification. The most commonly described nitrate-reducing Fe(II) oxidizers are mixotrophic and need a cosubstrate such as acetate to oxidize Fe(II) (1, 9, 16, 21, 22). Nitrite accumulation during growth of these strains raises the question of whether they are true (enzymatic) Fe(II) oxidizers, because the abiotic reaction of nitrite with Fe(II) has previously been demonstrated (for an example, see reference 11). Nevertheless, there are several strains for which it has been proposed to grow autotrophically with only Fe(II) as the electron donor and nitrate as the electron acceptor, e.g., *Thiobacillus denitrificans* (1), *Ferroglobus placidus* (18), *Pseudogulbenkiania* sp. strain 2002 (5), and other strains (23–29). However, these studies failed to provide unambiguous evidence for autotrophic nitrate reduction coupled to Fe(II) oxidation; for example, data demonstrating continuous transfers over long periods of time are missing. Nevertheless, nitrate reduction coupled to autotrophic Fe(II) oxidation has indeed been proven to exist in the environment and in lab cultures, as shown by Laufer et al. (3) in coastal marine sediments, by He et al. (30, 31), and by C. Tominski et al. in a companion manuscript (32) in the enrichment culture KS.

The enrichment culture KS (referred to here as culture KS) was isolated by Straub et al. (1) over 20 years ago and is currently probably the only culture for which autotrophic Fe(II) oxidation coupled to nitrate reduction has been demonstrated unequivocally for many cultivation transfers and many cell generations. Sequencing of the 16S rRNA gene and metagenome analyses revealed that the microbial consortium present in culture KS is dominated by an Fe(II) oxidizer, a species of the family *Gallionellaceae* (referred to here as *Gallionellaceae* sp.) related to the microaerophilic Fe(II) oxidizers *Sideroxydans* sp. ES-1 (96% 16S rRNA gene sequence similarity) and *Gallionella* sp. ES-2 (94% 16S rRNA gene sequence similarity) (30, 31). Other operational taxonomic units (OTUs) with lower relative sequence abundances were identified as heterotrophic bacteria, including, for example, *Bradyrhizobium*, *Rhodanobacter*, *Nocardioides*, and *Thiobacillus* (30, 31). All efforts to isolate the postulated Fe(II) oxidizer as an individual strain have so far been unsuccessful, suggesting some important role(s) carried out by the heterotrophic flanking community. One possible reason why *Gallionellaceae* sp. may need the heterotrophic community members is the lack of known nitric oxide and nitrous oxide reductases in its metagenome, which are needed to reduce NO further to N_2 (30). If the *Gallionellaceae* sp. reduces nitrate to only NO, the accumulated NO will need to be eliminated due to its toxic effect on cells (33). All flanking community members possess all the reductases necessary for complete denitrification (30), suggesting that the essential role of the heterotrophs is to reduce the toxic NO further to N_2, as previously suggested by Blöthe and Roden (31).

Interestingly, the community composition of culture KS changed in two separate

cultures of KS that were maintained over several years in two different laboratories at the University of Tuebingen, Germany (culture KS-Tueb) and the University of Madison, USA (culture KS-Mad) (30, 31). Relative sequence abundances of *Gallionellaceae* sp. differ from 96% in culture KS-Tueb to 42% in culture KS-Mad (30). In addition, the heterotrophic flanking community varied greatly between the two cultures, raising the question as to whether *Gallionellaceae* sp. needs a specific partner or any of the heterotrophic strains. Different efforts to isolate community members of culture KS yielded only heterotrophs that were not capable of autotrophic Fe(II) oxidation (1, 31). For the *Bradyrhizobium* spp., it was shown specifically that they are able neither to oxidize Fe(II) nor to reduce Fe(III) (unpublished data). Interestingly, recent metagenomic analysis of the heterotrophic community members showed the presence of different RuBisCO genes, suggesting that the heterotrophs should theoretically be able to grow by CO_2 fixation (30). During recent years, only the heterotrophic isolates have been characterized; however, on the whole, the community of enrichment culture KS has not been intensively investigated. So far, only few experiments on physiological characteristics of culture KS (as a whole) have been made; for example, it was shown that the culture can oxidize Fe(II)-containing minerals under anoxic conditions (34) or that it cannot grow with O_2 as an electron acceptor despite *Gallionellaceae* sp. being related to microaerophilic Fe(II) oxidizers (31, 35). However, the physiology of culture KS and the community dynamics during growth with different electron donors and acceptors have never been investigated. Furthermore, the potential for metabolic flexibility of culture KS compared to the single strains present in culture KS has not been determined. It is still unknown what the interactions and dependencies of the single community members are, how the community reacts to changing conditions such as those observed in the environment (e.g., a simultaneous increase in electron donors or alternating electron donors [AED]), and whether culture KS loses the ability to perform Fe(II) oxidation after long periods of Fe(II) limitation.

Therefore, the goals of our study were (i) to quantify relative abundances of the most prominent community members under different growth conditions using specific fluorescence in situ hybridization (FISH) probes, (ii) to characterize the metabolic flexibility of culture KS, and (iii) to monitor the ability of culture KS to perform Fe(II) oxidation under changing growth conditions. Hence, we followed Fe(II) or acetate oxidation coupled to nitrate reduction in comparison with cell numbers and relative cell abundances of *Gallionellaceae* sp. and *Bradyrhizobium* spp. Finally, we investigated the performance of Fe(II) oxidation by culture KS following the readdition of Fe(II) to the culture after Fe(II) oxidation stopped, when both Fe(II) and acetate were available as electron donors, when Fe(II) and acetate alternated as electron donors, and after several transfers of the culture on only acetate.

RESULTS

Growth with Fe(II), H_2, and organic compounds coupled to nitrate and O_2 reduction. Culture KS is an enrichment culture consisting of different community members: an autotrophic Fe(II) oxidizer (*Gallionellaceae* sp.) and several heterotrophic nitrate reducers (e.g., *Bradyrhizobium*, *Rhodanobacter*, *Nocardioides*). We found that culture KS can, in contrast to previous observations (31), be cultivated under microoxic conditions in gradient tubes and zero-valent iron (ZVI) plates (Fig. 1; Table 1). Culture KS showed anaerobic growth with $FeCl_2$, Fe(II)-EDTA, glucose, fructose, ribose, sucrose, acetate, lactate, fumarate, formate, propionate, butyrate, or pyruvate as the electron donor with nitrate as the electron acceptor (Table 1). No growth was observed when thiosulfate, citrate, H_2, or vivianite was provided as an electron donor in the presence of nitrate. Culture KS oxidized Fe(II) with nitrate and O_2 as electron acceptors, but not with N_2O (Table 2). Fe(III) minerals (ferrihydrite) were not reduced by KS in the presence of acetate, lactate, or H_2 as an electron donor. However, Fe(III)-EDTA was reduced in the presence of acetate.

Autotrophic growth (AG) with Fe(II). Culture KS was cultivated autotrophically with Fe(II) and nitrate. Because of slightly different initial substrate concentrations and

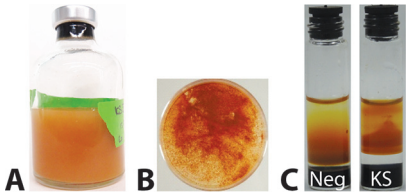

FIG 1 Growth of culture KS under anoxic conditions in serum bottles (A) and under microoxic conditions in a ZVI plate (B) and gradient tubes (C). Gradient tubes shown are an abiotic negative control (left) and a tube inoculated with culture KS (right).

lag phases, the results from two independent cultures showing the same trends were not given as an average. Instead, one representative data set is shown in Fig. 2 (the data for the second culture are shown in Fig. S1 in the supplemental material), but the results from the duplicate cultures are presented in the following sections as the range of the duplicates. Cell counts were performed for duplicate cultures following 4′,6-diamidino-2-phenylindole (DAPI) and FISH staining after hybridization with the culture KS-specific *Gallionellaceae* sp. (KS-Gal466) and *Bradyrhizobium* species (KS-Brady1249) probes (Fig. 3), which are described in reference 32.

Nitrate reduction and Fe(II) oxidation began immediately after inoculation and slowed down after 3 days [maximum Fe(II) oxidation rate, 2.0 ± 0.0 mM day^{-1}]. Culture KS consumed 7.0 to 8.8 mM Fe(II) (74 to 84%) and 1.5 to 1.9 mM nitrate (31 to 59%) within 10 days (Fig. 2A), yielding an $Fe(II)_{oxidized}$/$nitrate_{reduced}$ ratio of 4.5 to 4.8, close to the expected ratio for autotrophic growth of 5:1 (3, 31). DAPI-based total cell numbers increased from 4×10^4 to 5×10^4 cells/ml to 2×10^6 cells/ml after 10 days (Fig. 2A). *Gallionellaceae* sp. dominated culture KS with 58 to 83% relative abundance (Fig. 2B), and *Gallionellaceae* sp. cell numbers increased rapidly within the first 4 days, from 3×10^4 to 4×10^4 cells/ml to 1×10^6 cells/ml (Fig. 2A). Simultaneously, *Bradyrhizobium* spp. grew from 7×10^3 cells/ml to 3×10^5 cells/ml (Fig. 2A). However, *Bradyrhizobium* species cell numbers were 1 order of magnitude lower than total cell numbers, representing ca. 12 to 33% (Fig. 2B).

TABLE 1 Electron donors tested for growth with culture KS[a]

Substrate category and electron donor(s)	Electron acceptor	Growth	Source or reference(s)
Inorganic			
$FeCl_2$	Nitrate	+	This study
$FeSO_4$	Nitrate	+	1, 31
Fe(II)-EDTA	Nitrate	+	This study
Thiosulfate	Nitrate	−	This study
H_2	Nitrate	−	This study
Organic			
Acetate, lactate, fumarate, formate, propionate, butyrate, pyruvate	Nitrate	+	This study
Glucose, fructose, ribose, sucrose	Nitrate	+	This study
Citrate	Nitrate	−	This study
Minerals			
Biotite	Nitrate	+	34
Biotite	O_2	−	34
Biogenic magnetite	Nitrate	+	35
Microbially reduced goethite	Nitrate	+	35
Biogenic and synthetic siderite	Nitrate	+	35
Microbially reduced Fe(III)-rich subsoils	Nitrate	+	35
Chemically reduced NAu-2 smectite	Nitrate	+	34, 59
Vivianite	Nitrate	−	This study

[a]Growth is indicated as positive in experiments from this study if four subsequent transfers of the culture showed an increase in either turbidity or substrate consumption.

TABLE 2 Electron acceptors tested for growth with culture KS[a]

Electron acceptor(s)	Electron donor(s)	Growth	Source or reference(s)
Nitrate	See Table 1	+	1, 15, 31, 34, 35; this study
Nitrite	NA	+	15
5% N_2O	$FeCl_2$	−	This study
5% N_2O	NA	+	15
O_2	ZVI, FeS	+	This study
O_2	FeS	−	31
Ferrihydrite	Acetate	−	15; this study
Ferrihydrite	Lactate or H_2	−	This study
Fe(III)-EDTA	Acetate	+	This study
Thiosulfate, sulfate, sulfite, elemental sulfur, fumarate, malate	Acetate	−	15

[a]Growth is indicated as positive in experiments from this study if four subsequent transfers of the culture showed an increase in cell numbers and/or substrate consumption. NA, not available.

After nitrate reduction ceased at day 8 (with ca. 2.85 mM nitrate remaining), the culture shown in Fig. 2A was used to inoculate the alternating electron donor (AED) experiment and is named here culture AG (autotrophic growth). When additional 10 mM Fe(II) (but no nitrate) was added to the remaining culture after 10 days, Fe(II) oxidation and nitrate reduction started immediately, but with a lower Fe(II) oxidation rate of 1.2 ± 0.4 mM day^{-1}, and slowed down after 2 days (Fig. 2A). Twelve days after the additional Fe(II) spike (day 22 in the full experiment), 3.6 to 5.9 mM Fe(II) (40 to 52%) and 0.6 to 1.1 mM nitrate (34 to 38%) were consumed, yielding an $Fe(II)_{oxidized}$/$nitrate_{reduced}$ ratio of 5.4 to 5.5, again close to the expected ratio for autotrophic growth of 5:1 (3, 31). FISH-based cell counts and relative cell abundances of both *Gallionellaceae* sp. and *Bradyrhizobium* spp. showed only a slight increase in cell numbers during the second Fe(II) oxidation period. At day 22, when still ca. 5 to 6 mM total Fe(II) and 1 to 2 mM nitrate were remaining (Fig. 2A), an additional 5 mM Fe(II) (but no nitrate) was added. No further change was observed in either substrate concentrations or cell

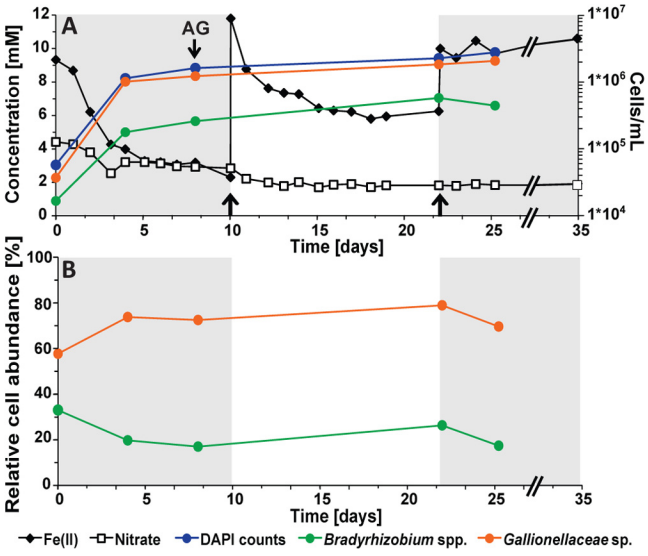

FIG 2 Substrate concentrations and cell numbers (A) and relative cell abundances (B) of the main community members of culture KS grown under autotrophic conditions with 10 mM Fe(II) and 4 mM nitrate. Cell numbers were plotted on a logarithmic scale. CARD-FISH-specific cell numbers (provided in panel A) were used to calculate the relative abundances of *Bradyrhizobium* spp. and *Gallionellaceae* sp. (B). After 10 and 22 days, Fe(II) was readded to the medium (black arrows). The gray shading indicates periods before and after addition of Fe(II). After 8 days, the culture was used as an inoculum for the alternating electron donors (AED) experiment and is named culture AG. Due to variations in the initial Fe(II) concentration and individual lag phases of different cultures, a representative data set is shown.

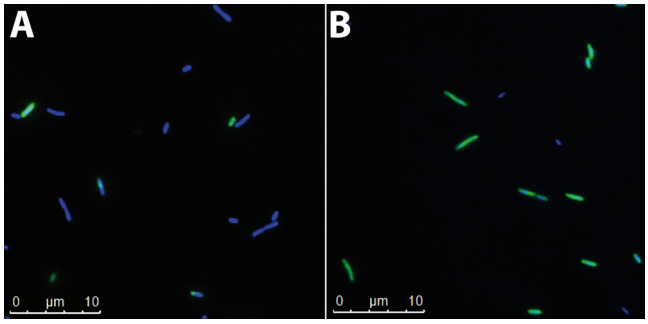

FIG 3 Fluorescence microscopy pictures of culture KS grown under mixotrophic conditions [10 mM Fe(II), 5 mM acetate, and 4 mM nitrate]. Shown are overlays of DAPI staining (all cells) and the specific CARD-FISH probes for *Gallionellaceae* sp. (KS-Gal466) (A) and *Bradyrhizobium* spp. (KS-Brady1249) (B).

numbers in the duplicate cultures within the following 13 days (day 35 in Fig. 2). Nitrite was not detected in any culture at any time point.

Mixotrophic growth with Fe(II), acetate, and nitrate. Culture KS was cultivated mixotrophically with Fe(II) and acetate as electron donors and nitrate as an electron acceptor. One representative data set from two parallel experiments of substrate concentrations, absolute cell numbers, FISH counts, and relative cell abundances of the main community members of culture KS is shown in Fig. 4 (second culture is shown in Fig. S2).

Generally, substrate conversion and growth under mixotrophic conditions can be divided into three phases (Fig. 4). In phase 1 (0 to 1.9 days), cell numbers of *Gallionellaceae* sp. remained stable, whereas *Bradyrhizobium* spp. increased in cell numbers while nitrate concentrations remained stable. Afterwards, the relative cell abundance of *Gallionellaceae* sp. decreased, although acetate concentrations were stable (day 2 to 6 in phase 2). Finally, acetate was consumed and *Gallionellacea*e sp. remained at a low relative cell abundance (phase 3). The $Fe(II)_{oxidized}$/$nitrate_{reduced}$ ratios were 1.1 to 1.3 mM Fe(II) oxidized and no nitrate consumed in phase 1, 2.7 to 3.1 in phase 2, and 0.9 to 1.2 in phase 3.

Fe(II) was immediately but slowly oxidized (1.2 ± 0.0 mM day^{-1}) over the 10 days (Fig. 4A). Nitrate reduction started after 3 days, and acetate oxidation started with a maximum rate of 0.7 ± 0.0 mM day^{-1} after a long lag phase of 6 days. Fe(II) oxidation under mixotrophic conditions was much slower than under autotrophic conditions, and nitrate was completely reduced. After 10 days, culture KS had consumed 7.7 to 7.8 mM Fe(II) (79.2 to 81.0%), 2.9 mM acetate (60.4 to 63.0%), and 4 mM nitrate (100%), yielding an $Fe(II)_{oxidized}$/$nitrate_{reduced}$ ratio of 1.9 and an $acetate_{oxidized}$/$nitrate_{reduced}$ ratio of 0.7.

During Fe(II) and acetate oxidation under mixotrophic conditions (over 10 days), total cell numbers increased from 6×10^4 cells/ml to 2×10^7 to 3×10^7 cells/ml (Fig. 4A). Interestingly, before a change in acetate concentrations was detected (at day 5), *Bradyrhizobium* spp. had already grown from 6×10^3 to 2×10^4 cells/ml to 2×10^6 to 3×10^6 cells/ml. Although Fe(II) was oxidized, *Bradyrhizobium* spp. overgrew *Gallionellaceae* sp. after 3 to 4 days (Fig. 4B). After 10 days, *Gallionellaceae* sp. reached cell numbers of 3×10^6 to 6×10^6 cells/ml, resulting in a decreasing relative cell abundance of *Gallionellaceae* sp. from 72% to between 4 and 10%, whereas *Bradyrhizobium* spp. became the dominant community member (up to 83%).

Alternating electron donor (AED) experiment. Culture KS was cultivated under conditions where the electron donor changed between Fe(II) (autotrophic) and acetate (heterotrophic) with nitrate as the electron acceptor from autotrophic (AED1) to heterotrophic (AED2), autotrophic (AED3), heterotrophic (AED4), autotrophic (AED5), and finally back to heterotrophic (AED6) conditions (Fig. 5, S3, and S4). The autotrophic culture AG was used as the inoculum for the alternating electron donor experiment (Fig. S3), meaning that culture AG was the same as AED1. All cultures were inoculated with 1% (vol/vol) from the previous culture.

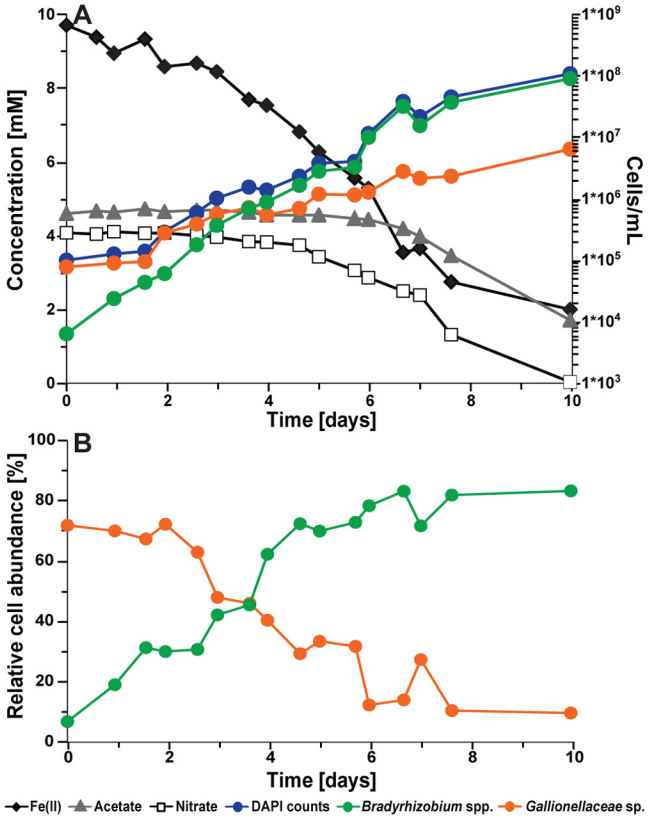

FIG 4 Substrate concentrations and cell numbers (A) and relative abundances (B) of the main community members of culture KS grown under mixotrophic conditions with 10 mM Fe(II), 5 mM acetate, and 4 mM nitrate. Cell numbers were plotted on a logarithmic scale. CARD-FISH-specific cell numbers (provided in panel A) were used to calculate the relative abundances of *Bradyrhizobium* spp. and *Gallionellaceae* sp. (B). Due to variations in initial Fe(II) concentration and individual lag phases of different cultures, a representative data set is shown.

Fe(II) oxidation under autotrophic conditions showed similar behaviors (rates and extent of oxidation) after intermediate growth on acetate, i.e., Fe(II) oxidation always started immediately after inoculation and ceased after 8 to 10 days (Fig. 5A). Culture KS oxidized 5.9 to 10.1 mM Fe(II) in the AG (i.e., AED1), AED3, and AED5 cultures. Interestingly, Fe(II) oxidation became more efficient (higher extent of oxidation) after each intermediate cultivation on acetate from 71% in AG to 74% in AED3 and finally 87% in AED5. In AG, AED3, and AED5, 1.3 to 2.2 mM nitrate was reduced (34 to 54%), yielding $Fe(II)_{oxidized}$/$nitrate_{reduced}$ ratios of 4.5 to 4.8, 4.6 to 5.7, and 4.7 to 5.9 in AG, AED3, and AED5, respectively, in all three cases close to the expected ratio for autotrophic growth of 5:1.

Under heterotrophic conditions, culture KS typically started to consume acetate and nitrate after a lag phase of 5 to 6 days (Fig. 5A). At the end of the incubation phase (12 days for AED2 and AED4, 25 days for AED6), 3.2 to 4.6 mM acetate and 3 to 5 mM nitrate were consumed, resulting in $acetate_{oxidized}$/$nitrate_{reduced}$ ratios of 0.9 to 1.8, 0.9 to 1.0, and 1.0 in AED2, AED4, and AED6, respectively. The amount of acetate oxidized depended on the initial nitrate concentration. Acetate oxidation became less efficient, going from 91% (AED2) to 69% (AED6) after each intermediate cultivation with Fe(II) as the electron donor. After acetate and nitrate consumption stopped in phase AED2, this culture was used in addition as the inoculum for the survival experiment (SE). Nitrite was not observed in any of the cultures AED1 to AED6.

Generally, cell numbers were lower when culture KS was grown autotrophically than under heterotrophic conditions (Fig. 5A). DAPI-based total cell numbers increased from 4×10^4 to 8×10^5 cells/ml to 2×10^6 to 3×10^6 cells/ml in AG and AED3 under

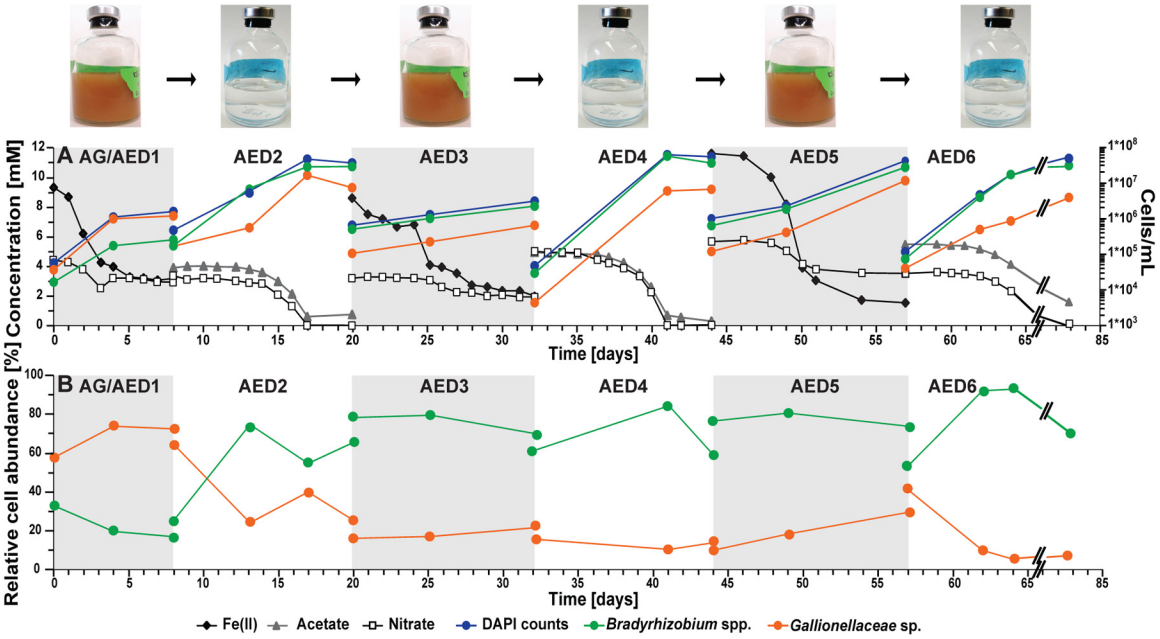

FIG 5 Substrate concentrations and cell numbers (A) and relative cell abundances (B) of the main community members in the alternating electron donor (AED) experiment, in which culture KS was cultivated with either Fe(II)/nitrate (autotrophic, gray boxes) or acetate/nitrate (heterotrophic, white boxes). (A) Cell numbers were plotted on a logarithmic scale. (B) CARD-FISH-specific cell numbers were used to calculate the relative abundances of *Bradyrhizobium* spp. and *Gallionellaceae* sp. Culture AG/AED1 is the same as described for Fig. 2. Due to variations in initial substrate concentrations and individual lag phases of different cultures, a representative data set is shown.

autotrophic conditions (Fig. 6A). Slightly higher cell numbers were reached in AED5 (4×10^6 to 4×10^7 cells/ml), which coincided with the highest percentage of oxidized Fe(II) (87%). Under heterotrophic conditions, total cell numbers increased from 5×10^4 to 5×10^5 cells/ml to 4×10^7 to 2×10^8 cells/ml (AED2, AED4, AED6). *Gallionellaceae* sp. dominated culture KS in AG under autotrophic growth conditions (representing on average 73% of the bacterial population). However, the relative cell abundance of *Gallionellaceae* sp. decreased rapidly under heterotrophic conditions (AED2), from 73% abundance to 9 to 26% abundance, and *Bradyrhizobium* spp. remained dominant over the whole experiment in all heterotrophic and even in the subsequent autotrophic cultures (AED2 to AED6) (Fig. 5B). Under the subsequent autotrophic conditions, the relative cell abundance of *Gallionellaceae* sp. did increase slightly but only to a maximum of 29% (AED5).

Survival experiment (SE). We investigated whether *Gallionellaceae* sp. could survive several subsequent transfers under heterotrophic conditions with only acetate as the electron donor and if the *Gallionellaceae* sp. was still able to oxidize Fe(II) under autotrophic conditions. Culture KS was cultivated heterotrophically four times on acetate (survival experiment 1 [SE1] to SE4) (Fig. 6A to C, S3, and S5A to C). Culture AED2 was used as the inoculum, meaning that AED2 was the same as SE1. Heterotrophic cultures SE1, SE2, SE3, and SE4 were then transferred back to Fe(II)/nitrate to test for autotrophic growth (these cultures were named AHG [after heterotrophic growth]) (Fig. 6D to F, S3, and S5D to F).

With every transfer on acetate, the length of the lag phase, i.e., the time before acetate consumption started, decreased (Fig. 6A). In cultures SE1 to SE4, 3.2 to 3.8 mM acetate (79.5 to 82.1%, 1.2 ± 0.1 mM day^{-1}) was oxidized and 2.8 to 3.8 mM nitrate was reduced (96.7 to 100%), yielding $acetate_{oxidized}/nitrate_{reduced}$ ratios of 1.0 to 1.2, 0.9 to 1.0, and 0.9 to 1.4 for cultures SE2, SE3, and SE4, respectively (SE1 is the same as AED2 described above). DAPI- and FISH-based total cell numbers and those of *Bradyrhizobium* spp. increased from 9×10^3 to 5×10^5 cells/ml to 3×10^7 to 7×10^7 cells/ml, respectively, in SE1 to SE4 during heterotrophic growth (Fig. 6B). *Bradyrhizobium* spp.

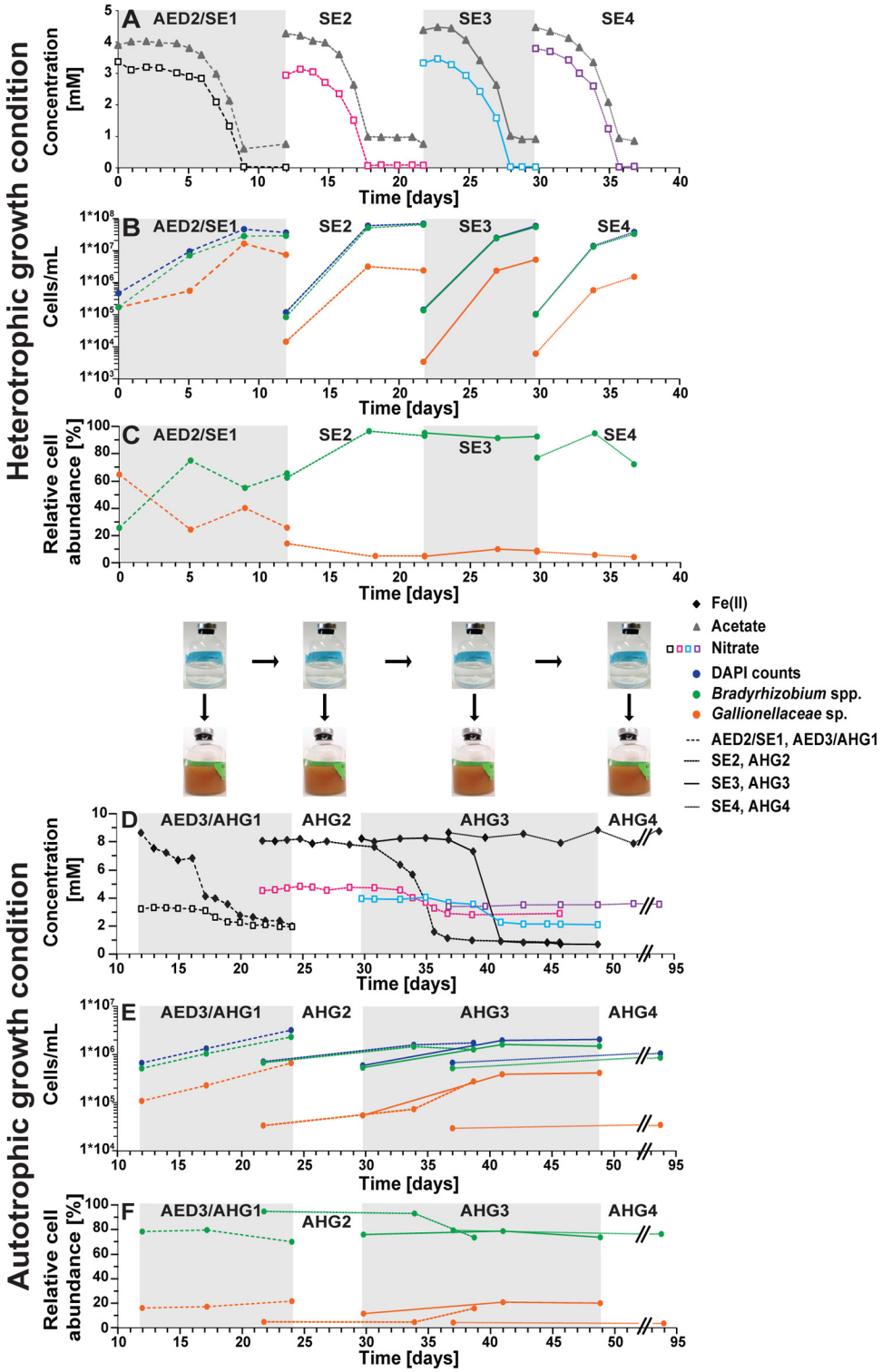

FIG 6 Substrate concentrations (A, D), cell numbers (B, E), and relative cell abundances (C, F) for the main community members in the survival experiment (SE) in which culture KS was cultivated first several times with acetate/nitrate (heterotrophic, cultures SE1 to SE4) (A to C) and then transferred back to autotrophic conditions [Fe(II)/nitrate, cultures AHG1 to AHG4] (D to F). (B, E) Cell numbers were plotted on a logarithmic scale. (C, F) CARD-FISH-specific cell numbers were used to calculate the relative abundances of *Bradyrhizobium* spp. and *Gallionellaceae* sp. Cultures AED2/SE1 and AED3/AHG1 are the same as described for Fig. 5. Due to variations in initial substrate concentrations and individual lag phases of different cultures, a representative data set is shown.

became dominant in the SE1 culture and remained dominant over the whole experiment (Fig. 6C). *Gallionellaceae* sp. also grew under heterotrophic conditions but had cell numbers 1 order of magnitude lower than cell numbers of *Bradyrhizobium* spp. (Fig. 6B).

When transferring the heterotrophic cultures back to Fe(II)/nitrate, 5.9 to 7.5 mM Fe(II) (71.1 to 91.5%) was oxidized and 1.3 to 1.9 mM nitrate (38.3 to 47.6%) was reduced, yielding $Fe(II)_{oxidized}$/$nitrate_{reduced}$ ratios of 4.6 to 5.7, 4.0 to 4.5, and 4.1 to 4.2 in AHG1, AHG2, and AHG3, respectively (Fig. 6D). No Fe(II) oxidation was observed in AHG4 (even after 55 days of incubation), which was inoculated from SE4, although the same *Gallionellaceae* sp. cell numbers were reached in SE4 as in the previous cultures SE1, SE2, and SE3 (Fig. 6B). For the first three back-transfers to Fe(II)/nitrate, after each cultivation on acetate, the lag phases for Fe(II) oxidation increased, meaning that AHG1 showed the fastest and AHG3 the slowest Fe(II) oxidation. Although Fe(II) was oxidized under autotrophic conditions in AHG1 to AHG3, *Bradyrhizobium* spp. dominated culture KS in all cultures (Fig. 6F). *Bradyrhizobium* species and total cell numbers increased only a maximum of 1 order of magnitude, from 5×10^5 to 1×10^6 cells/ml to 1×10^6 to 3×10^6 cells/ml in cultures AHG1, AHG2, and AHG3 (Fig. 6E). Cell numbers of *Gallionellaceae* sp. followed the same trend as *Bradyrhizobium* spp. but were 1 order of magnitude lower.

DISCUSSION

Continuous autotrophic Fe(II) oxidation. Culture KS is currently the only nitrate-reducing Fe(II)-oxidizing culture for which it has been explicitly demonstrated that it can be continuously cultivated under autotrophic conditions for more than 20 years, demonstrating that the microbial community in this culture couples nitrate reduction to autotrophic Fe(II) oxidation. This implies that Fe(II) oxidation is enzymatically catalyzed (probably by the *Gallionellaceae* sp.) in culture KS and is not an abiotic side reaction via nitrite as it has been suggested for mixotrophic NRFO cultures (11). This conclusion is supported by the following evidence: (i) the continued cultivation of culture KS without an added organic cosubstrate for more than 2 decades (31); (ii) the absence of nitrite accumulation independent from the cultivation conditions (1, 36); (iii) the active incorporation of [^{13}C]bicarbonate by *Gallionellaceae* sp. during growth under autotrophic conditions with only Fe(II) as the electron donor (32); (iv) the same proposed Fe oxidase MtoA and Cyc2 homologs that were found in the microaerophilic Fe(II) oxidizers *Sideroxydans* sp. ES-1 and *Gallionella* sp. ES-2 have also been found in the genome of the *Gallionellaceae* sp. present in KS (30); and (v) cells from culture KS did not become encrusted during Fe(II) oxidation, and the minerals formed by KS differed from those formed during the abiotic oxidation of Fe(II) by nitrite (11, 36). Although all these observations strongly support that culture KS is an autotrophic Fe(II)-oxidizing culture with an enzymatic Fe(II) oxidation mechanism that showed consistent Fe(II) oxidation when transferred into fresh medium with Fe(II) and nitrate, we also made the interesting observation that a further addition of Fe(II) to an existing KS culture that still contained nitrate showed limited Fe(II) oxidation after the first Fe(II) addition and no further Fe(II) oxidation after the second addition (Fig. 2). The reason for this remains unknown, and we can only speculate that this could be due to the buildup of toxic concentrations of metabolites, the lack of certain trace metals or nutrients [due to sorption to or coprecipitation with formed Fe(III) minerals (37, 38)], or the formation of Fe(II) minerals that cannot be oxidized by culture KS. Although culture KS can oxidize the Fe(II)-containing minerals biotite, magnetite, and siderite (34, 35), it is unable to oxidize vivianite, a poorly soluble Fe(II)-phosphate [$Fe_3(PO_4)_2 \cdot 8H_2O$] that is formed immediately after the addition of $FeCl_2$ to the phosphate-containing medium (39) and remains even after Fe(II) oxidation has ceased in culture KS (36). Vivianite precipitation might also explain why Fe(II) oxidation in culture KS was always incomplete, although sufficient nitrate as an electron acceptor was present, which has also been observed previously (1, 31).

Metabolic flexibility of culture KS. As a community, culture KS can oxidize and reduce a wide range of different inorganic and organic electron donors and acceptors. For instance, nitrate can be replaced by small amounts of oxygen as an electron acceptor by culture KS, which is due to the fact that the heterotrophic community members are known to be facultative anaerobes (for examples, see references 40, 41, and 42) and the dominant community member *Gallionellaceae* sp. is closely related to known microaerophilic Fe(II) oxidizers. No growth by culture KS was observed with N_2O as the electron acceptor and Fe(II) as the electron donor, which was expected because *Gallionellaceae* sp. lacks the nitric oxide (NO) and nitrous oxide (N_2O) reductases in its genome (30).

Acetate is commonly used as an additional electron donor for the cultivation of mixotrophic NRFO (for examples, see references 1, 9, and 16). Fe(II) oxidation under mixotrophic conditions by culture KS with acetate as an additional electron donor was shown by Nordhoff et al. (36), but it was not known how the culture KS community develops when electron donors alternate or are present simultaneously. In the present study, we demonstrated a shift in the KS community composition, i.e., *Bradyrhizobium* spp. overgrew *Gallionellaceae* sp. on day 4 (Fig. 4) although Fe(II) oxidation started and proceeded before any change in acetate concentration was observed. It has to be noted, however, that already a concentration of 125 μM acetate provides the same number of electrons as 1 mM Fe(II) and small changes in acetate concentrations might not be detected because of the sensitivity of the acetate quantification method. Both Fe(II) (1.2 mM day^{-1}) and acetate (0.7 mM day^{-1}) oxidations were slower under mixotrophic conditions than under autotrophic [2 mM Fe(II) day^{-1}] and heterotrophic (1.2 mM acetate day^{-1}) growth conditions. One explanation could be that *Gallionellaecae* sp. was using both Fe(II) and acetate as substrates at the same time.

In this study, we demonstrated growth of culture KS, especially *Bradyrhizobium* spp. and *Gallionellaceae* sp., under heterotrophic conditions with acetate as the sole electron donor (Fig. 5 and 6). Until now, it was not clear if *Gallionellaceae* sp. can grow and survive under heterotrophic conditions without Fe(II) even for more transfers and a longer time. We found that the Fe(II) oxidizer reached similar cell numbers under autotrophic, mixotrophic, and heterotrophic conditions as well as when electron donors [Fe(II) and acetate] alternated or when culture KS was cultivated for several transfers with acetate as the sole electron donor, raising the question as to which electron donor is preferred by the *Gallionellaceae* sp. Our study clearly showed that the *Gallionellaceae* sp. is able to use acetate as an alternative electron and energy source to Fe(II), these findings confirming the results from a parallel nanoSIMS (nanoscale secondary ion mass spectrometry) study in our laboratory, reported in the companion paper (32)—a very surprising finding considering that in the *Gallionellaceae* sp. genome the genes for acetate uptake were not detected (30). Interestingly, for the related strains *Sideroxydans* sp. ES-1 and *Gallionella* sp. ES-2, neither acetate oxidation nor Fe(II) oxidation coupled to nitrate reduction has been shown, suggesting that the *Gallionellaceae* sp. in culture KS may represent a novel genus within the *Gallionellaceae*.

Another interesting observation that we made was that although the *Gallionellaceae* sp. Fe(II) oxidizer in culture KS survived Fe(II)-free conditions at least for four transfers under heterotrophic conditions, it lost its ability to oxidize Fe(II) after the fourth transfer with only acetate. A similar loss of Fe(II) oxidation ability was observed in the "Madison culture" by Eric Roden (personal communication). However, in order to clarify if the loss of function is associated with a loss of genes involved in Fe(II) oxidation, acquisition of mutations, or loss of genes involved in Fe stress response, resequencing of a culture KS metagenome after 4 or 5 transfers with only acetate as an electron donor will be necessary. In contrast, when culture KS was cultivated not four times but only once under heterotrophic conditions and transferred back to autotrophic conditions, *Gallionellaceae* sp. was always overgrown by *Bradyrhizobium* spp. under heterotrophic conditions, but the *Gallionellaceae* sp. maintained its ability to oxidize Fe(II) (Fig. 5 and 6). The observed activity and dominance of the *Bradyrhizobium* spp. under dynamic

environmental conditions., i.e., under changing substrate composition and concentrations, show its ecological fitness, not only under heterotrophic conditions but also when culture KS was cultivated for only one transfer under heterotrophic conditions, when conditions were reverted back to autotrophic in the next transfer, or under mixotrophic conditions, when Fe(II) was oxidized but *Bradyrhizobium* spp. overgrew *Gallionellaceae* sp. already after 3 to 4 days.

Environmental relevance of culture KS as an Fe(II)-oxidizing microbial community. We were able to demonstrate that culture KS is able to metabolize Fe(II) and a variety of organic substrates and is thus able to adapt to fluctuating and dynamic environmental conditions. Even when the community composition changed and *Bradyrhizobium* became the dominant community member, Fe(II) was still oxidized by *Gallionellaceae* sp. This was true even when culture KS was being exposed to Fe(II)-limiting or even Fe(II)-free incubation conditions (for up to three transfers with only acetate as the electron donor) before being switched back to Fe(II)/nitrate. Several environmental studies provide evidence for the importance of these microorganisms in nature. Sequences of the family *Gallionellaceae* were found in an aquifer (43), a high-CO_2 subsurface geyser (44), wetland soils (45), heavy-metal-rich mine water discharge (46), an inactive seafloor hydrothermal sulfide chimney (47), and Fe(II)-oxidizing bacterial mats (48), highlighting the general importance of chemolithoautotrophic Fe(II) oxidation and a connection between the biogeochemical cycles of N, C, and Fe. More evidence of the existence and importance of autotrophic nitrate-reducing Fe(II) oxidizers came from a marine sediment microcosm study (3) in which a ratio of 0.22 to 0.28 $nitrate_{reduced}/Fe(II)_{oxidized}$ was detected during Fe(II) oxidation and concomitant nitrate reduction. These ratios are very similar to our calculated ratios of 0.21 to 0.24 $nitrate_{reduced}/Fe(II)_{oxidized}$ in culture KS under autotrophic conditions. In addition, recent studies demonstrated that Fe(II) oxidation coupled to denitrification can serve as a biological nitrogen remover from wastewater (49), constructed wetlands (50), and contaminated ground water (51) and is also used as a treatment of nitrate-containing wastewaters and simultaneously as a bioprocess for Fe biorecovery (52).

In summary, our data demonstrated that culture KS is a dynamic microbial community, responding to changes in the available organic and inorganic substrates. Isolation and physiological characterization of the individual KS community members would give more information about the mechanisms of Fe(II) oxidation in this culture. Additionally, other isolation strategies (e.g., separation of individual cells using laser tweezers) could be used to isolate the Fe(II) oxidizer of culture KS. To identify the dependence of the main community members *Gallionellaceae* sp. and *Bradyrhizobium* spp. on each other and possible cross-feeding and interactions between these two strains, stable-isotope probing (e.g., DNA-SIP) or nanoSIMS analyses are necessary.

MATERIALS AND METHODS

Sources of microorganisms, microbial growth medium, and growth conditions. The enrichment culture KS (culture KS-Tueb, referred to here as culture KS) was isolated from freshwater sediment in Bremen (1) and has been cultivated in our laboratory for several years. For cultivation of culture KS, serum bottles containing anoxic freshwater medium (pH 6.8 to 7.0) buffered with 22 mM bicarbonate were used. The medium was prepared anoxically with an N_2-CO_2 (90:10 [vol/vol]) headspace as described in detail by Hegler et al. (53). For the growth experiments, different electron donors and acceptors were tested. These electron donors were separately used: 10 mM $FeCl_2$, 2 mM vivianite, and 5 mM (each) Fe(II)-EDTA, thiosulfate, acetate, lactate, fumarate, formate, propionate, butyrate, pyruvate, glucose, fructose, ribose, sucrose, citrate, and H_2 (10% or 80%) with 4 mM nitrate as the electron acceptor. As electron acceptors, 4 mM nitrate and 5% N_2O were tested with 10 mM $FeCl_2$ as the electron donor. In addition, 5 mM ferrihydrite [$Fe_{8.2}O_{8.5}(OH)_{7.4}{\cdot}3H_2O$] (54) was tested as the electron acceptor with 5 mM (each) acetate, lactate, or 80% H_2 as electron donor, while 5 mM Fe(III)-EDTA was tested as the electron acceptor with 5 mM acetate as the electron donor. For mixotrophic growth conditions, 10 mM $FeCl_2$, 5 mM acetate, and 4 mM nitrate were used. During AED and the *Gallionellaceae* sp. survival experiments, growth conditions were changed from autotrophic (10 mM $FeCl_2$ and 4 mM nitrate) to heterotrophic (5 mM acetate and 4 mM nitrate). All cultures were inoculated with 1% (vol/vol) of an autotrophically grown preculture and incubated at 28°C in the dark. Growth experiments were conducted in duplicate (AG and mixotrophic conditions) or triplicate (AED and SE).

Vivianite [$Fe_3(PO_4)_2{\cdot}8H_2O$] was synthesized by adding 10 mM $FeCl_2$ to several bottles of the anoxic freshwater medium containing 4.4 mM phosphate, followed by the precipitation of a whitish-greyish

precipitate. After 1 h, the mineral suspension was centrifuged (room temperature [RT], 15 min, 4,000 rpm) and gently transported into an anoxic glove box. The supernatant was removed, and the vivianite was washed and again centrifuged with anoxic medium in the glove box to remove any Fe(II) bound to the mineral phase three times. Finally, the centrifuged vivianite was suspended in 20 ml anoxic freshwater medium, and the Fe(II) concentration was determined after acidic dissolution with the ferrozine assay.

For testing microaerophilic growth, zero-valent iron (ZVI) plates and gel-stabilized gradient tubes were used (with ZVI and Fe^{2+}, respectively, being the only electron source for microaerophilic growth), as described in detail by Laufer et al. (4). Instead of the artificial seawater medium (ASW), modified Wolfe's mineral medium (MWMM) was used (55).

Analytical methods. For quantification of Fe(II) and Fe(III) concentrations, we used the revised ferrozine protocol for nitrite-containing samples described by Klueglein and Kappler (9) and Schaedler et al. (56). Briefly, 100 μl of culture suspension was withdrawn anoxically with a syringe and mixed with 900 μl of 40 mM sulfamic acid. Sulfamic acid reacts with the nitrite present and therefore prevents abiotic oxidation of Fe(II) by reactive nitrogen species formed during sample acidification (9). The purple ferrozine-Fe(II) complex was quantified at 562 nm using a microtiter plate reader (FlashScan 550; Analytik Jena, Germany). Ferrozine measurements were done in triplicates. Acetate samples were taken in an anoxic glove box (N_2 atmosphere), centrifuged (maximum speed, RT, 10 min), and stored at −20°C before they were quantified by high-performance liquid chromatography (HPLC; class VP with refractive index detector [RID] 10A and photo-diode array detector SPD-M10A VP detectors; Shimadzu, Japan); the precolumn was a Microguard cation H cartridge; the main column was an Aminex HPX-87H ion exclusion column (300 mm by 7.8 mm; Bio-Rad, Austria); the eluent was 5 mM H_2SO_4 in double-distilled water (ddH_2O). Nitrate and nitrite samples were taken in an anoxic glove box (N_2 atmosphere), centrifuged (maximum speed, RT, 10 min), and stored under anoxic conditions at 5°C until they were quantified using a continuous-flow analyzer with a dialysis membrane for Fe removal to prevent side reactions during analysis (Seal Analytical, Norderstedt, Germany). For continuous flow analysis, nitrate is reduced to nitrite with hydrazine sulfate and quantified photometrically with N-1-naphthyl ethylenediamine at 520 nm. Maximum rates of microbial Fe(II) oxidation, acetate oxidation, and nitrate reduction were calculated from the steepest slope between three subsequent data points of Fe(II), acetate, and nitrate concentrations, respectively.

CARD-FISH. The relative cell abundances of *Gallionellaceae* sp. and *Bradyrhizobium* spp. during growth under different conditions were quantified following catalyzed amplification reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with the horseradish peroxidase (HRP)-labeled oligonucleotide probes KS-Gal466 (including the unlabeled helper oligonucleotides KS-Gal431-helper, KS-Gal449-helper, and KS-Gal485-helper) and KS-Brady1249 as described in reference 32. As positive and negative controls, probe Beta42a for *Betaproteobacteria* (modified from the probe described in reference 57) and probe NON338 (modified from reference 58), respectively, were used. All probes were ordered as HRP conjugates (biomers.net GmbH, Ulm, Germany).

Sample preparation, hybridization, and cell counts were performed as described in reference 32. Briefly, samples were fixed with 1% paraformaldehyde for 4 to 16 h at 4°C, washed, and stored at −20°C. Cultures containing Fe were treated with an oxalate solution to dissolve the Fe minerals. Afterwards, the cells were filtered onto Isopore polycarbonate membrane filters and embedded in 0.2% low-melting-point agarose. *Gallionellaceae* sp. cells were permeabilized with proteinase K (≧0.71 μg ml^{-1}) at RT for 15 min. *Bradyrhizobium* species cells were permeabilized with lysozyme (10 mg ml^{-1}) at 37°C for 1 h. For both probes, 35% formamide and 46°C were used as hybridization conditions.

After hybridization and DAPI staining (1 μg ml^{-1}), membrane filters were embedded in Prolong Gold and incubated at RT for 24 h in the dark before the slides were analyzed using fluorescence microscopy (Leica DM 5500 B [Leica Microsystems]). Images were taken at a magnification of ×1,000 with a Leica DFC 360 FX camera using the Leica application suite advanced fluorescence software (2.6.0.766). Cell counts were performed on duplicate cultures, counting at least 500 cells per sample. The cell numbers were calculated from the filtered culture volume and the counted filter area.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02173-17.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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We declare that we have no conflict of interest.

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