Systematic and Applied Microbiology 45 (2022) 126306

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.com/locate/syapm

‘*Candidatus* ferrigenium straubiae’ sp. nov., ‘*Candidatus* ferrigenium bremense’ sp. nov., ‘*Candidatus* ferrigenium altingense’ sp. nov., are autotrophic Fe(II)-oxidizing bacteria of the family *Gallionellaceae*

Yu-Ming Huang [a,b], Natalia Jakus [a,b], Daniel Straub [a,c], Konstantinos T. Konstantinidis [d], Nia Blackwell [a], Andreas Kappler [b,e], Sara Kleindienst [a,*]

[a] *Microbial Ecology, Center for Applied Geoscience, University of Tuebingen, 72076 Tuebingen, Germany*
[b] *Geomicrobiology, Center for Applied Geoscience, University of Tuebingen, 72076 Tuebingen, Germany*
[c] *Quantitative Biology Center (QBiC), University of Tuebingen, 72076, Germany*
[d] *School of Civil and Environmental Engineering, School of Biological Sciences, and Center for Bioinformatics and Computational Genomics, Georgia Institute of Technology, Atlanta, GA 30332, United States*
[e] *Cluster of Excellence: EXC 2124: Controlling Microbes to Fight Infections, Tübingen, Germany*

ARTICLE INFO

Article history:
Received 8 July 2021
Revised 6 February 2022
Accepted 7 February 2022

Keywords:
Nitrate-reducing Fe(II) oxidation
Denitrification
Gallionellaceae
‘*Candidatus* Ferrigenium straubiae’
‘*Candidatus* Ferrigenium bremense’
‘*Candidatus* Ferrigenium altingense’

ABSTRACT

Iron(II) [Fe(II)] oxidation coupled to denitrification is recognized as an environmentally important process in many ecosystems. However, the Fe(II)-oxidizing bacteria (FeOB) dominating autotrophic nitrate-reducing Fe(II)-oxidizing enrichment cultures, affiliated with the family *Gallionellaceae*, remain poorly taxonomically defined due to lack of representative isolates. We describe the taxonomic classification of three novel FeOB based on metagenome-assembled genomes (MAGs) acquired from the autotrophic nitrate-reducing enrichment cultures KS, BP and AG. Phylogenetic analysis of nearly full-length 16S rRNA gene sequences demonstrated that these three FeOB were most closely affiliated to the genera *Ferrigenium*, *Sideroxydans* and *Gallionella*, with up to 96.5%, 95.4% and 96.2% 16S rRNA gene sequence identities to representative isolates of these genera, respectively. In addition, average amino acid identities (AAI) of the genomes compared to the most closely related genera revealed highest AAI with *Ferrigenium kumadai* An22 (76.35–76.74%), suggesting that the three FeOB are members of this genus. Phylogenetic analysis of conserved functional genes further supported that these FeOB represent three novel species of the genus *Ferrigenium*. Moreover, the three novel FeOB likely have characteristic features, performing partial denitrification coupled to Fe(II) oxidation and carbon fixation. Scanning electron microscopy of the enrichment cultures showed slightly curved rod-shaped cells, ranging from 0.2-0.7 μm in width and 0.5–2.3 μm in length. Based on the phylogenetic, genomic and physiological characteristics, we propose that these FeOB represent three novel species, ‘*Candidatus* Ferrigenium straubiae’ sp. nov., ‘*Candidatus* Ferrigenium bremense’ sp. nov. and ‘*Candidatus* Ferrigenium altingense’ sp. nov. that might have unique metabolic features among the genus *Ferrigenium*.

Introduction

Ferrous iron [Fe(II)] oxidation can be mediated by specific microorganisms and plays a crucial role in biogeochemical nitrogen, carbon, oxygen and sulfur cycles of natural and engineered ecosystems [1], such as freshwater sediment [2–4], marine coastal sediment [5,6] and constructed wetlands [7]. Fe(II)-oxidizing bacteria (FeOB) are capable of oxidizing both Fe(II)-minerals precipitated in the solution and dissolved Fe(II) as electron donors. Studies of FeOB living at circumneutral pH have demonstrated that these organisms flourish within the zone in which ferrous ions are stable, i.e. at microoxic to anoxic conditions at which abiotic Fe(II) oxidation is limited enough for microorganisms to compete [8,9]. Most known FeOB are members of *Proteobacteria* and are grouped into three physiological types, depending on the electron acceptor

Abbreviations: NRFeOx, nitrate-reducing Fe(II)-oxidizing; FeOB, Fe(II)-oxidizing bacteria; MAG, metagenome assembled genome; AAI, average amino acid identity; ANI, average nucleotide identity; *Ca.*, *Candidatus*; acc. no., accession number.

* Corresponding author at: Microbial Ecology, Center for Applied Geosciences Eberhard-Karls-University Tuebingen, Schnarrenbergstrasse 94-96, 72076 Tuebingen, Germany.
E-mail address: sara.kleindienst@uni-tuebingen.de (S. Kleindienst).

https://doi.org/10.1016/j.syapm.2022.126306
0723-2020/

and energy source used for Fe(II) oxidation: light-dependent (photoferrotrophs), O_2-dependent (microaerophiles) and nitrate-reducing [2,10,11].

Many of the common neutrophilic Fe(II)-oxidizing bacteria belong to the family *Gallionellaceae*, which are divided into eight defined genera. Four of these are represented entirely by FeOB: *Ferrigenium, Ferriphaselus, Gallionella* and *Sideroxydans.* These bacteria typically oxidize Fe(II) under microoxic conditions [12–21] and a few of them, unclassified *Gallionellaceae* spp., were suggested to couple Fe(II) oxidation to partial denitrification under anoxic conditions [2,8,22–25]. Additionally, some of the FeOB in the *Gallionellaceae* family were reported to perform carbon fixation [8,12–21], as they are very often inhabiting organic carbon depleted environments such as aquifers [8], geysers and high-CO_2 subsurface wetland soils [26], inactive seafloor hydrothermal sulfide chimneys [27] as well as mine water discharges [28]. Despite the genetic potential of these bacteria and the broad variety of habitats that members of the family *Gallionellaceae* occupy, there is no reported isolate of neutrophilic autotrophic nitrate-reducing Fe(II)-oxidizing (NRFeOx) bacteria. However, members of the family *Gallionellaceae* were found to dominate microbial communities of known neutrophilic autotrophic NRFeOx enrichment cultures [2,24,25].

So far there are only three published examples of stable autotrophic NRFeOx enrichment cultures. Two of the cultures were obtained from freshwater sediments in Bremen, northern Germany (cultures KS and BP) [2,24], and one was obtained from an anoxic groundwater monitoring well in Altingen, southern Germany (culture AG) [25]. Recent metagenomic analysis revealed that all of these unclassified *Gallionellaceae* spp. in the three enrichment cultures share many common features. They possess the genes encoding putative Fe(II) oxidases such as Cyc2 and/or MtoAB, and genes involved in denitrification such as nitrate reductase (*narGHI*), nitrite reductase (*nirK/S*) and/or nitric oxide reductase (*norBC*) [22–24,29]. The bacteria were also found to possess the large subunit ribulose-1,5-bisphosphate carboxylase-oxygenase gene (*rbcL*), a key gene for carbon dioxide fixation during the Calvin–Benson–Bassham (CBB) cycle [22–24,29,30]. Thus, they likely have the ability to perform autotrophic Fe(II) oxidation coupled to partial denitrification. Based on nearly full-length 16S rRNA gene sequence (∼1460 bp) analysis, most closely related isolated species of the dominating *Gallionellaceae* spp. present in these cultures are *Ferrigenium kumadai* An22, *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2. Despite elaborate cultivation attempts, these three FeOB *Gallionellaceae* spp. have so far not been isolated [22,24,25,30]. Furthermore, the exact phylogenetic placement and taxonomic description for these FeOB in the *Gallionellaceae* family remains unresolved.

An increasing number of available genomes derived from metagenomics studies of so far uncultured members of the family *Gallionellaceae*, sharing the potential of coupling Fe(II) oxidation to partial denitrification, shows the importance of classifying these new taxa of FeOB [8,19,31]. In addition, the key FeOB of the only stable NRFeOx cultures that exist to date (i.e. cultures KS, BP and AG) and that are currently frequently used as model systems to study autotrophic nitrate reduction coupled to Fe(II) oxidation remain to be classified. Moreover, the advancement of methods in (meta)genomics complement traditional approaches and promote the assessment of the existing taxonomic system [32,33]. For example, a widely applied approach is to use the category of '*Candidatus*' to propose putative taxa [34]. These yet-to-be isolated microorganisms do not meet all requirements of the International Code of Nomenclature of Prokaryotes [35]; however, there is sufficient evidence to justify their classification through (meta)genomic data [33,35–38]. Here, we describe phylogenetic, genomic and phenotypic analyses to suggest the designation of the three novel FeOB species within the family *Gallionellaceae*.

Materials and methods

Cultivation and isolation strategies

Culture KS, named after Kristina Straub who reported the first study on this culture [2] (dominated by '*Ca.* Ferrigenium straubiae' strain KS) originated from a freshwater ditch in Bremen, Germany. Since then, culture KS has been transferred for more than 20 years under autotrophic conditions with 1–10% (v/v) inoculum [22,23,30]. Several cultivation and isolation techniques have been used, e.g. cultivation under microoxic conditions in gradient tubes and zero-valent iron (ZVI) plates [30], or growth with different substrates under anoxic conditions in serum bottles, such as $FeCl_2$, Fe(II)-EDTA and $FeSO_4$ as the electron donor with nitrate as the electron acceptor [2,30,39]. Culture BP, named after Bremen Pond, (dominated by '*Ca.* Ferrigenium bremense' strain BP) originated from a freshwater pond in the backyard of the Max Planck Institute for Marine Microbiology, Bremen, Germany in 2015 [24]. Since then, culture BP has been transferred for more than 2 years (>20 times/year since 2018) under autotrophic conditions with 4–10% (v/v) inoculum. Both culture KS and culture BP were grown in 25 mL unfiltered, bicarbonate-buffered medium, containing 10 mM $FeCl_2$, 4 mM $NaNO_3$, vitamins, and trace elements with a final pH of 6.9 to 7.2 and ca. 33 mL anoxic headspace (N_2/CO_2 ratio was 90/10) in 58 mL serum bottles, closed with black butyl rubber stoppers [39,40], and incubated in the dark at 28 °C. Culture AG, named after Altingen Groundwater, (dominated by '*Ca.* Ferrigenium altingense' strain AG) originated from an anoxic groundwater monitoring well in Altingen, southern Germany [25]. Since then, culture AG has been transferred for more than 3 years (>20 times/year since 2018) under autotrophic conditions with 25 mL anoxic headspace (N_2/CO_2 ratio was 90/10), unfiltered, bicarbonate-buffered medium, containing 2 mM $FeCl_2$, 2 mM $NaNO_3$, vitamins and trace elements with a final pH of 6.9 to 7.2 in 58 mL serum bottles, closed with black butyl rubber stopper with 10% (v/v) inoculum [25]. So far, the revival after the storage with 10%, 15% and 20% (v/v) glycerol could not be achieved, and, thus, all three cultures (KS, BP and AG) currently need to be maintained by regular transfer.

PacBio long-read 16S rRNA gene amplicon sequencing

DNA extraction for culture KS, culture BP and culture AG was reported previously [24,25]. PacBio Sequel SMRT long-read amplicon sequencing was performed at the Helmholtz Zentrum München, Germany. DNA amplification was conducted twice using two rounds of PCR with primers universal for bacterial 16S rRNA genes, tailed with PacBio universal sequencing adapters (universal tags) and 5′ amino modifiers (27F gcagtcgaacatgtagctgactcaggtcacAGRGTTYGATYMTGGCTCAG, 1492R tggatcacttgtgcaagcatcacatcgtagRGYTACCTTGTTACGACTT) (Biomers.net, Ulm, Germany) to amplify the nearly full-length 16S rRNA genes from the genomic DNA extracted of all three cultures, respectively [24,25]. The PCR amplification protocol was described in detail previously [24,25]. For the SMRTbell library preparation [41], the SMRTbell Template Prep Kit (PacBio biosciences, California, USA) was applied according to the user's manual instructions.

11,688 circular consensus sequencing reads were obtained for culture KS, 20,635 for culture BP, and 28,979 for culture AG. Subsequently, reads were analyzed with DADA2 v1.10.0 [42,43] in R v3.5.1 [44] by sequentially orienting reads and removing primers, filtering (no ambiguous nucleotides and maximum 2 expected errors) and trimming (1000 bp to 1600 bp read length), dereplicating sequences, learning error rates, removing chimera de novo and finally assigning taxonomy to the detected sequences based on

SILVA v132 [45]. Lastly, 8 amplicon sequencing variants (ASVs) with 5,543 total counts were obtained for culture KS, 25 ASVs with 7,831 total counts for culture BP and 36 ASVs with 17,575 total counts for culture AG. The relative abundance of the five most abundant ASVs of each culture is shown in Table S1.

Metagenome sequencing, assembly and annotation

Approximately 1 μg of DNA was used for library preparation with the TruSeq DNA PCR-Free Kit from Illumina without modifications and libraries were sequenced on the Illumina NovaSeq 6000 platform to generate paired-end (2 × 150-bp) reads by CeGaT, Tuebingen, Germany. For culture KS, 55.8 and 48.8 Gbp (giga base pairs) raw sequences were generated for two samples, 27.5 Gbp for culture BP and 23.8 Gbp for culture AG. Nanopore sequencing (Oxford Nanopore Technologies; ONT) using PromethION platform on culture KS was performed by the NGS Competence Center Tuebingen (NCCT) at the University of Tuebingen, Germany, and produced 53 Gbp in 5 million reads. ONT sequencing on culture AG, was performed by GenXone Inc, Suchy Las, Poland, and yielded 13 Gbp in 2.5 million reads. Short and long read quality control, (hybrid) assembly, and metagenome assembled genome binning was performed with nf-core/mag v1.0.0 (https://doi.org/10.5281/zenodo.3589528) [46,47] with Nextflow [48] using containerized software with singularity v3.0.3 [49]. Short read quality was assessed with FastQC v0.11.8 [50], quality filtering and Illumina adapter removal was performed with fastp v0.20.0 [51], and reads mapped with Bowtie2 v2.3.5 [52] to the PhiX genome (Enterobacteria phage WA11, GCA_002596845.1, ASM259684v1) were removed. Long read quality was assessed with NanoPlot v1.26.3 [53], adapter trimming was done with Porechop v0.2.3_seqan2.1.1 (https://github.com/rrwick/Porechop), *Escherichia* virus Lambda (PRJNA485481, GCA_000840245.1) contamination was removed with Nanolyse v1.1.0 [53], and quality filtering was performed with Filtlong v0.2.0 (https://github.com/rrwick/Filtlong) using short reads (with default setting for culture AG but using nf-core/mag parameters "--longreads_keep_percent 75 --longreads_length_weight 1" for culture KS). Finally, processed short and long reads (culture KS and culture AG) were assembled with metaSPAdes v3.13.1 [54] or short reads (culture BP) with MEGAHIT v1.2.7 [55]. The assemblies were evaluated with QUAST v5.0.2 [56]. MAGs were binned with MetaBAT2 v2.13 [57] aided by the sequencing depth, checked for their completeness and contamination with BUSCO v3.0.2 [58] using 148 near-universal single-copy orthologs of bacteria (http://busco.ezlab.org/v3/datasets/bacteria_odb9.tar.gz) selected from OrthoDB v9 [59], summary statistics were obtained with QUAST for each MAG. The assembled metagenomes and MAGs were uploaded to the Joint Genome Institute's Integrated Microbial Genome and Microbiome Expert Review (IMG/MER) pipeline (IMGAP) for annotation (available online at https://img.jgi.doe.gov/cgi-bin/mer/main.cgi; [60]).

Metagenome assembly genome (MAGs) analysis

FeGenie [61] was used to search for potential Fe(II) oxidation genes, e.g. *cyc2, mtoAB, mofA* [62], and the IMG [60] and the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) [63,64] databases were used for obtaining genomes and 16S rRNA gene sequences of closely related taxa. The basic local alignment search tool (BLAST) [65] was used to compare the nearly full-length 16S rRNA gene sequences of the *Gallionellaceae* spp. of cultures KS, BP and AG to those gene sequences of *Gallionellaceae* spp. that were isolated or identified in environmental samples.

Phylogenetic tree construction

The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model [66]. This analysis involved 14 nearly full-length 16S rRNA gene nucleotide sequences and 9 concatenated house-keeping, protein-coding, amino acid gene sequences. There were a total of 1622 and 4529 positions in the final dataset, respectively. The tree with the highest log likelihood was selected with 1000 bootstraps of nucleotide and amino acid sequences. The percentage of trees in which the associated taxa clustered together was indicated next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X [67]. The house-keeping genes, i.e. RNA polymerase α subunit (*rpoA*), DNA gyrase α subunit (*gyrA*), protein translocase subunit (*secA*), isoleucyl-tRNA synthetase (*ileS*), rho termination factor (*rho*) and translation initiation factor IF-2 (*infB*), were selected via the gene sequence availability of the top three closest related isolated genomes and other physiological related species (i.e. FeOB of the family *Gallionellaceae* and NRFeOx bacteria), to generate a concatenated maximum likelihood phylogenetic tree [16,68–70].

Average nucleotide identity (ANI) and average amino acid identity (AAI)

The average nucleotide identity (ANI) and alignment fraction (AF) was analyzed via the online tool IMG/MER Pairwise ANI (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi?section=ANI&page=pairwise) [60,71,72]. Average amino acid identity (AAI) was conducted via the online tool AAI calculator, developed by the Environmental Microbial Genomics Laboratory (enve-omics lab) at the Georgia Institute of Technology (Kostas lab) (http://enve-omics.ce.gatech.edu/) [73].

Scanning electron microscopy

For scanning electron microscopy (SEM) analysis, samples of the enrichment cultures KS, BP and AG were taken during the mid Fe(II)-oxidizing phase (after 3 days of incubation under autotrophic conditions) and at the end of the Fe(II) oxidation phase (after 6 days of incubation under autotrophic conditions). SEM samples were fixed in 2.5% glutaraldehyde for three hours on ice, by adding 100 μl of a 25% glutaraldehyde solution directly to 900 μl of centrifuged cell suspension in culture medium. After fixation, the samples were centrifuged (1 min, 2348x g) to concentrate the cells and approximately 900 μl of supernatant was removed and replaced by MQ H_2O to wash out the glutaraldehyde from the sample. This procedure was repeated twice. Then, 25 μl of each sample was dropped onto Poly-l-Lysine coated cover glass slides [coated each with 75 μl 0.1% Poly-L-Lysine solution (PLANO, Wetzlar, item number 18026) and air dried over night before usage], placed in a 12-well plate. The plate was then covered with the plate lid and left for 15 minutes for the samples to settle. In the following, the samples were dehydrated by a graded ethanol series (30%, 70%, 95% for 5 min each; 2 × 100% for 30 min). In a final step, the samples were dipped into hexamethyldisilazane (HMDS) in two separated continuous-flow analysis vials in sequence for 30 seconds each and left to dry on filter paper afterwards. The cover glass slides were then fixed onto aluminum stubs with carbon tape (PLANO, Wetzlar, item numbers G301 & G3347) and sputter-coated with ~ 8 nm Pt by use of a BAL-TEC SCD 005. The SEM examination was performed at a Crossbeam 550L FIB-SEM (Zeiss,

Oberkochen, Germany) using the InLens or SESI detector at an acceleration voltage of 2 kV and working distances of 4.0–5.3 mm.

Data availability and figure illustration

The heat maps were constructed via R v3.6.1 and its graphical user interface RStudio (https://www.R-project.org/ and http://www.rstudio.com/) [74,75].

The datasets presented in this study can be found in online repositories: SRR14879643 for culture KS including '*Ca*. Ferrigenium straubiae' strain KS, SRR13504099 for culture BP including '*Ca*. Ferrigenium bremense' strain BP and SRR10568922 for culture AG including '*Ca.* Ferrigenium altingense' strain AG (Table 1). The three nearly full-length 16S rRNA gene sequences of strain KS, strain BP and strain AG are provided in the supplementary file. The IMG metagenome IDs for cultures KS, BP and AG are 3300040739, 3300036710 and 3300041015 and the corresponding accession numbers of the MAGs of '*Ca.* Ferrigenium straubiae', '*Ca.* Ferrigenium bremense' and '*Ca.* Ferrigenium altingense' in IMG are 2878407288, 2831290873 and 2860363623, respectively (Table 1). The corresponding JGI GOLD analysis project IDs and names are Ga0439409, "Combined Assembly of Lab enriched freshwater sediment microbial communities from Bremen, Germany"; Ga0394452, "Lab enriched sediment microbial communities from pond in Bremen, Germany - BP MEGAHIT" and Ga0436356, "Freshwater bacterial community from groundwater monitoring well in Altingen, Germany - NRFeOx_Alt1". The NCBI genome acc. nos. for '*Ca.* Ferrigenium straubiae', '*Ca.* Ferrigenium bremense' and '*Ca.* Ferrigenium altingense' are JAHQXD000000000, JAGRPI000000000 and JAHRYS000000000, respectively (Table 1).

Results and discussion

The novel strains represent dominant taxa of autotrophic NRFeOx enrichment cultures

Based on the near full-length 16S rRNA gene sequence analysis, all three *Gallionellaceae* spp., strain KS, strain BP and strain AG, dominated the autotrophic NRFeOx enrichment cultures KS, BP and AG, with $\geq$ 95%, $\geq$71% and $\geq$ 50% relative sequence abundance, respectively [22–25]. Highest abundance of these strains was observed during the exponential phase of growth, i.e. during the most active Fe(II) oxidation and nitrate reduction phase. All three NRFeOx cultures were cultivated under anoxic autotrophic conditions with $FeCl_2$ and $NaNO_3$ [2,24,25] as electron donor and acceptor (Fig. 1), respectively. The average $Fe(II)_{oxidized}$/$nitrate_{reduced}$ stoichiometric ratio was 4.28 in culture KS, 3.4 in culture BP and 5.0 in culture AG [23–25,30].

Both culture KS and culture BP originate from freshwater sediments collected in Bremen from a ditch [2] and a pond [24], respectively, while culture AG was obtained from an aquifer [25]. The geochemistry at the habitat of enrichment culture KS's origin was neither stated in the first publication [2] nor in subsequent publications. However, since the ditch of culture KS's origin is close by and connected to the pond of culture BP's origin, the geochemistry might be similar. The physicochemical conditions at the area of the sampling site, where culture BP was obtained, were measured in 2017 as follows; temperature: 11.7–11.9 °C, pH: 6.0–6.3, oxygen saturation: 8.5–74.3%, conductivity: 32.5–36.8 µS/m, $Fe(II)_{pore\ water}$: 0.324–4.85 µM, $Fe(III)_{pore\ water}$: 2.51–1 µM, nitrate: 0.714 µM, total organic carbon (TOC) in sediment: 0.16–0.45% (wt%) and dissolved organic carbon (DOC) in pond water: 0.6–0.7 mg/L [76]. The physicochemical parameters of the groundwater accessed via the monitoring well in Altingen were continuously monitored during observation periods (2004–2018) and the average values were calculated as follows; temperature: 12.5 ± 1.6 °C, pH: 7.1 ± 0.1, dissolved O_2: 0.1 ± 0.1 mg/L, conductivity: 885.1 ± 75.1 µS/cm, nitrate concentration: 0.02 ± 0.01 mM and DOC: 1.2 ± 0.3 mg/L [25].

Despite the different isolation habitats of the three cultures, all three FeOB unite a unique feature within the family *Gallionellaceae*, i.e. the ability to grow under autotrophic anoxic conditions and perform partial denitrification coupled to Fe(II) oxidation [2,24,25]. This unique capability differentiates them from all the isolated strains of the family *Gallionellaceae*, which typically perform microaerophilic Fe(II) oxidation using oxygen as electron acceptor [2,13,15,18,24,25,77,78].

Phylogenetic and genome (MAG) identity analysis of FeOB within the Gallionellaceae

In order to classify the three *Gallionellaceae* spp., strain KS, strain BP and strain AG, we performed phylogenetic analysis, using nearly full-length 16S rRNA gene sequences and available MAGs in the family *Gallionellaceae* (Fig. 2 and Fig. 3). Strain KS, strain BP and strain AG were closely related to each other and formed a novel clade within the phylogenetic tree (Fig. 2). The most closely related isolated species was *Ferrigenium kumadai*, which is a microaerophilic Fe(II)-oxidizing bacterium and was cultivated using gradient tubes [13,79]. *Ferrigenium kumadai* has 96.45%, 96.17% and 96.24% of nearly full-length 16S rRNA gene sequence identity with strain KS, strain BP and strain AG, respectively (Fig. 3) [23–25]. The second most closely related isolated species of strain KS and strain BP was *Sideroxydans lithotrophicus* ES-1, with 95.9% and 95.30% identity of nearly full-length 16S rRNA gene sequences, respectively (Fig. 3) [23,24]. The second most closely related isolated species of strain AG was *Gallionella capsiferriformans* ES-2, sharing 96.17% of nearly full-length 16S rRNA gene sequence identity (Fig. 3) [25]. As with *Ferrigenium kumadai*, both *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2 are microaerophilic Fe(II)-oxidizing bacteria [18] and have been cultivated using gradient tubes. While *Ferrigenium kumadai* An22 originated from rice paddy soil [13], *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2 were both from a groundwater-fed iron seep [18]. The three *Gallionellaceae* spp., strain KS, strain BP and strain AG, revealed 16S rRNA gene similarities of 97.06–97.88% among each other, indicating that they represent distinct species within the same genus (Fig. 3).

In addition, using metagenome binning methods, nearly complete and high-quality genomes (MAGs) were obtained for the *Gallionellaceae* spp. strain KS, strain BP and strain AG, with a completeness of 99.3%, 93.2% and 89.9%, respectively (Table 1) [23–25]. Further, we compared the AAI and ANI of all five isolated *Gallionellaceae* spp. to the MAGs of strain KS, strain BP and strain AG. Based on the AAI and ANI results, the closest related, isolated strains affiliated with the genera *Ferrigenium*, *Sideroxydans* and *Gallionella* (Fig. 3, Fig. S1). All three of the novel *Gallionellaceae* spp., i.e. strain KS, strain BP and strain AG, displayed AAI values in the range between 76.35% to 76.74% for *Ferrigenium kumadai* as well as 67.12% to 70.97% for *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2 (Fig. 3). The AAI alignment fraction coverages of the three novel *Gallionellaceae* spp. and closest related strains were all higher than 48% (Table S2). As the AAI of the three novel *Gallionellaceae* spp. compared to *Ferrigenium kumadai* were higher in comparison to *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2, this indicates that all three strains (i.e. strain KS, strain BP and strain AG) belong to the same genus (AAI threshold > 65%), postulated as genus *Ferrigenium*, but repre-

Table 1
Description table of '*Candidatus* Ferrigenium straubiae' sp. nov., '*Candidatus* Ferrigenium bremense' sp. nov. and '*Candidatus* Ferrigenium altingense' sp. nov.

Genus name	*Candidatus* Ferrigenium	*Candidatus* Ferrigenium	*Candidatus* Ferrigenium
Species name	*straubiae*	*bremense*	*altingense*
Genus status	-	-	-
Genus etymology	-	-	-
Type species of the genus	*Ferrigenium kumadai* An22	*Ferrigenium kumadai* An22	*Ferrigenium kumadai* An22
Specific epithet	*straubiae*	*bremense*	*altingense*
Species status	sp. nov.	sp. nov.	sp. nov.
Species etymology	strau'bi.ae. N.L. gen. n. *straubiae*, of Straub, honouring Dr. Kristina Straub who enriched, cultured and studied culture KS in 1993–1996.	bre.men'se. M.L. neut. adj. *bremense*, originating from Bremen, Germany.	al.tin.gen'se. N.L. neut. adj. *altingense*, originating from Altingen, Germany.
Description of the new taxon and diagnostic traits	Found in freshwater habitats. Cells are rod shaped. Fimbriae and flagella were not observed. Cells are 0.8–2.2 μm long and 0.2–0.7 μm wide. *In silico* genome analysis indicated that the species possesses potential Fe (II) oxidation genes: *cyc2, mtoAB, mofA*; denitrification genes: *narGHIJ, nirK/S*; and the carbon fixation gene: *rbcL*.	Found in freshwater habitats. Cells are rod shaped. Fimbriae and flagella were not observed. Cells are 1.1–2.3 μm long and 0.2–0.6 μm wide. *In silico* genome analysis indicated that the species possesses potential Fe (II) oxidation genes: *cyc2, mofA (distant homologs)*; denitrification genes: *nirK/S, norBC*; and the carbon fixation gene: *rbcL*.	Found in freshwater habitats. Cells are rod shaped. Fimbriae and flagella were not observed. Cells are 0.5–1.8 μm long and 0.2–0.6 μm wide. *In silico* genome analysis indicated that the species possesses potential Fe (II) oxidation genes: *cyc2, mofA (distant homologs)*; denitrification genes: *narGHIJ, nirK/S, norBC*; and the carbon fixation gene: *rbcL*.
Country of origin	Germany	Germany	Germany
Region of origin	Bremen, Bremen	Bremen, Bremen	Altingen, Baden-Wuerttemberg
Source of isolation	freshwater sediment	freshwater sediment	aquifer, groundwater monitoring well
Sampling date (dd/mm/yyyy)	1993	25/09/2015	12/09/2017
Latitude (xx°xx′xx″N/S)	-	53°06′36.7″N	48° 33′ 47.52″N
Longitude (xx°xx′xx″E/W)	-	8°50′48.6″E	8° 53′ 59.28″W
Altitude (meters above sea level)	N/A	N/A	378.5
16S rRNA gene accession nr.	IMG gene ID: 2878408845	Sequence listed in supplementary file	IMG gene ID: 2860363887
IMG Genome ID	2878407288	2831290873	2860363623
NCBI Genome accession number	JAHQXD000000000	JAGRPI000000000	JAHRYS000000000
Genome status	incomplete (MAG) completeness: 99.3%	incomplete (MAG) completeness: 93.2%	incomplete (MAG) completeness: 89.9%
Genome size (bp)	2,659,708	2,446,084	2,180,025
GC mol%	60.13%	58.92%	57.97%
Number of strains in study	1	1	1
Source of isolation of non-type strains	N/A	N/A	N/A
Information related to the Nagoya Protocol	N/A	N/A	N/A
Designation of the Type Strain	strain KS	strain BP	strain AG
Strain Collection Numbers	N/A	N/A	N/A

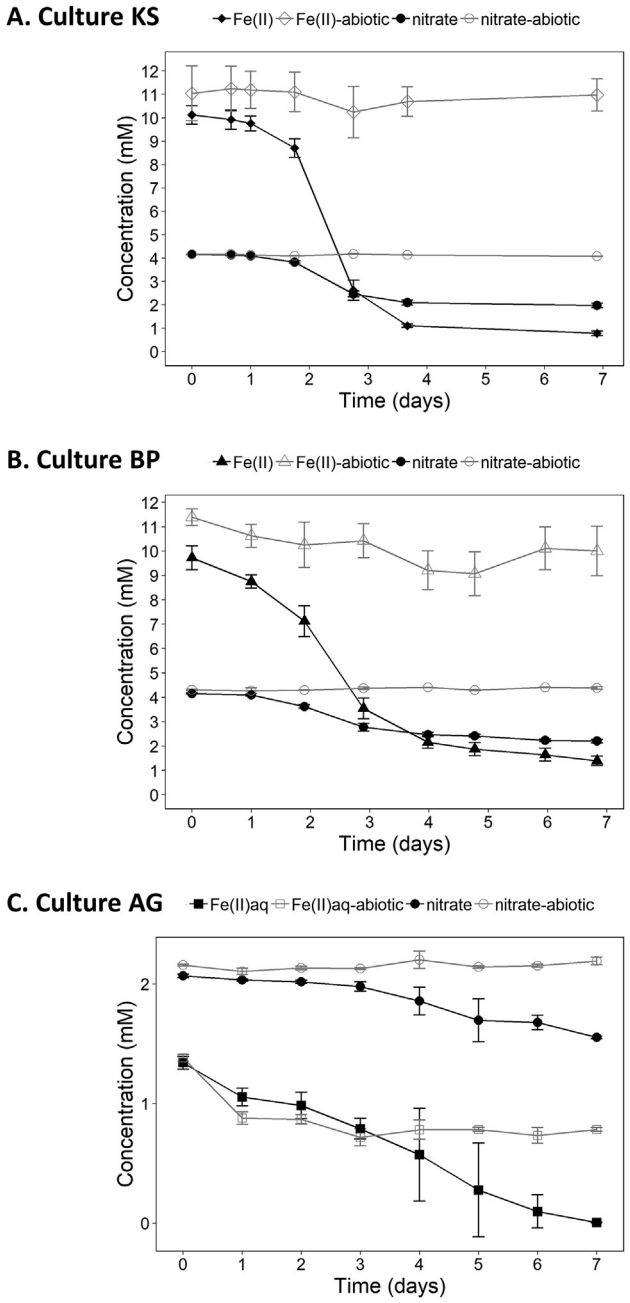

Fig. 1. Average Fe(II) and nitrate concentration over time for the enrichment cultures (A) KS, (B) BP and (C) AG. The chemistry data of culture KS and culture AG were replotted from publications by Huang *et al.*, 2021 [23] and Jakus *et al.*, 2021 [25], respectively.

sent different species (AAI threshold < 95%) [73]. It has to be noted, however, that the AAI values of *Ferrigenium kumadai*, *Gallionella capsiferriformans* ES-2 and *Sideroxydans lithotrophicus* ES-1 (70–71%) are at the border of being distinguishable genera and, thus, their taxonomic relationships could be revisited in the future (not a goal of our study). These results also revealed that it is crucial to take into consideration multiple lines of evidence to classify the new taxa of FeOB. The AAI results were consistent with the 16S rRNA gene-based results mentioned above which supported that the new taxa of FeOB are most closely related to *Ferrigenium kumadai*. Moreover, these novel species, i.e. strain KS, strain BP and strain AG, have AAI values in the range of 82.51% to 85.02% compared to each other (Fig. 3), suggesting that they are more closely related to each other than to any described isolate. This result suggests that they should be designated as distinct species of the same genus. As for ANI, a threshold of ANI $\geq$ 96.5% (alignment fraction: AF $\geq$ 60%) was proposed for the same species [72,80]. Our results showed that the ANI values between the three novel *Gallionellaceae* spp. and the most closely related isolate, *Ferrigenium kumadai*, range between 81.12% to 81.38% (Fig. S1), while the ANI to *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2 are in the range of 75.05% to 79.47% (Fig. S1).

For more evidence of the genealogical position, the amino acid sequences of house-keeping genes, which encode the RNA polymerase α subunit (*rpoA*), DNA gyrase α subunit (*gyrA*), protein translocase subunit (*secA*), isoleucyl-tRNA synthetase (*ileS*), rho termination factor (*rho*) and translation initiation factor IF-2 (*infB*), were selected to generate a concatenated maximum likelihood phylogenetic tree (Fig. 4) [16,68–70]. The phylogenetic analysis of concatenated house-keeping genes indicated that strain KS, strain BP and strain AG were closely affiliated to *Ferrigenium kumadai* An22, and were more distantly related to other isolated members of *Gallionellaceae* genera, a proposed neutrophilic mixotrophic nitrate-reducing FeOB, i.e. *Thiobacillus denitrificans* [81] and an acidophilic aerobic FeOB, i.e. *Acidithiobacillus ferrooxidans* (Fig. 4) [82].

Taken together the results of nearly full-length 16S rRNA gene identity and phylogeny as well as AAI, ANI and house-keeping gene phylogenetic analyses indicated strain KS, strain BP and strain AG, belong to the same genus, postulated as *Ferrigenium*, but represent different species.

Putative metabolic and physiological features

The metagenomic results showed that all three *Gallionellaceae* MAGs of strain KS, strain BP and strain AG have at least one putative Fe(II) oxidation gene, which could accept the electrons from the Fe(II) substrate, i.e. *cyc2* and *mofA* for all three MAGs and *mtoA* in the MAG of strain KS (Fig. 5) [22,24,29,62]. Among these genes, *cyc2*, *mtoA* and *mofA* were detected at transcript level and MofA was detected at protein level in culture KS [23]. On the other hand, both transcript and protein of *cyc2* were detected in culture BP [24]. To date, a metagenome was constructed and analyzed for culture AG [29], while metatranscriptomic and metaproteomic analysis for culture AG have not yet been done.

As for the electron acceptor, the genes for a partial denitrification pathway detected in MAGs of the strain KS, strain BP and strain AG were different. For strain KS, the genes, transcripts and proteins encoding nitrate reductase (NarGHI) and nitrite reductase (NirK/S) were detected under autotrophic conditions (Fig. 5) [23]. For strain BP, the genes, transcripts and proteins encoding NirK/S were detected and the genes and transcripts encoding nitric oxide reductase (NorBC) were detected under autotrophic conditions (Fig. 5) [24]. The genes encoding NarGHI, NirK/S and NorBC were all detected in strain AG (Fig. 5) [29]. These results indicated that strain KS, strain BP and strain AG probably have the ability to perform only partial denitrification. As previously suggested [23,24,29], they might require other denitrifiers to complete the denitrification pathways.

An alternative electron accepting pathway was speculated to be oxidative phosphorylation (e.g., via respiratory chain complexes I-V) [22,83,84]. The genetic potential for energy generation via respiratory chain complexes I-V were detected in strain KS, strain BP and strain AG [22,24,29]. Interestingly, the detection of homologous genes encoding the cbb_3- and aa_3- type cytochrome *c* oxidases of complex IV indicates that strain KS, strain BP and strain AG could have the ability to respire oxygen [23,24,29]. Some microoxic growth conditions were tested for culture KS [30] as well as culture AG [25], i.e. in gradient tubes or zero valent iron plates, but a stable growth was not yet observed.

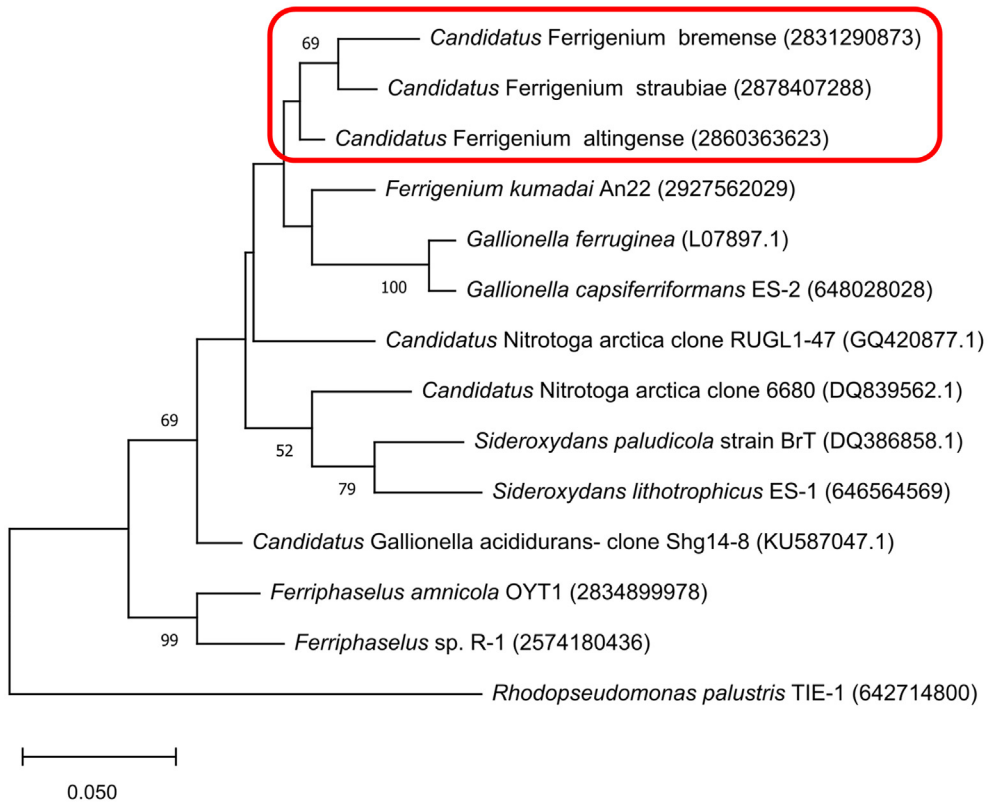

Fig. 2. Phylogenetic tree of seven isolated *Gallionellaceae* spp. and six metagenome-assembled genomes (*Candidatus* species), using available full or nearly full-length 16S rRNA gene sequences and calculated on 1000 replicates using the Maximum Likelihood method. The red box highlights the species classified in this study. The scale bar represents branch lengths measured by the number of substitutions per site. The numbers in brackets show the identifier of each gene in the IMG or NCBI databases.

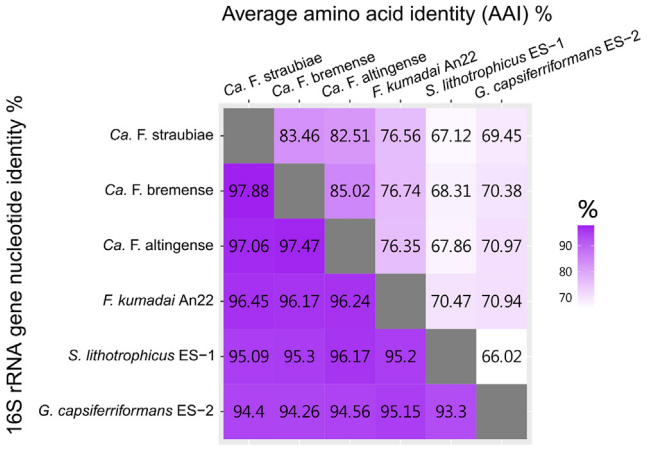

Fig. 3. Nearly full-length 16S rRNA gene identity and average amino acid identity (AAI) of strains from '*Candidatus* Ferrigenium straubiae' (strain KS) sp. nov., '*Candidatus* Ferrigenium bremense' (strain BP) sp. nov. and '*Candidatus* Ferrigenium altingense' (strain AG) sp. nov. compared to their top three closely related isolated strains affiliating with the genera *Ferrigenium*, *Sideroxydans* and *Gallionella*. Recommended thresholds: nearly full-length 16S rRNA gene sequence identity: genus > 95%, species > 98.6%; AAI: genus 65–95%, species ≥ 95% [38].

Moreover, due to the detection of the essential gene for carbon fixation, i.e. the large subunit ribulose-1,5-bisphosphate carboxylase-oxygenase gene (*rbcL*), it was proposed that strain KS, strain BP and strain AG have the ability to fix carbon to grow under autotrophic conditions [22–24,29,85]. The dominance of strain KS, strain BP and strain AG in the different NRFeOx enrichment cultures, i.e. culture KS, culture BP and culture AG, respectively, was likely caused by the ability to fix carbon by these three strains. In culture KS, carbon fixation by strain KS was additionally reported using nanoscale secondary ion mass spectrometry (NanoSIMS) to monitor the incorporation of ^{13}C-labeled bicarbonate [85] and the transcript and protein encoding the essential carbon fixation enzyme, RbcL, were detected for strain KS under autotrophic conditions [23]. For strain BP, the transcript of *rbcL* was also detected [24], while there are currently no transcript and protein data available for culture AG.

Morphology of the three Gallionellaceae spp.

In this study, we analyzed the cell morphology by scanning electron microscopy (SEM) (Fig. 6). It should be noted, however, that the timepoint for SEM sampling was determined based on relative abundance of nearly full-length 16S rRNA gene sequence to capture the most dominant strains (i.e. Fe(II) oxidizers); the relative abundance of strain KS, strain BP and strain AG in the three cultures KS, BP and AG were ≥95%, ≥71% and ≥50% [24,25]. For culture KS, the cell sizes (averaged for 17 cells) ranged from 0.8-2.2 μm in length and 0.2–0.7 μm in width (Table 2). For culture BP, the cell sizes (averaged for 27 cells) ranged from 1.1-2.3 μm in length and 0.2–0.6 μm in width (Table 2). As for culture AG, the cell sizes (averaged for 15 cells) ranged from 0.5-1.8 μm in length and 0.2–0.6 μm in width (Table 2). In the SEM images of the three enrichment cultures, the observed cells were either rod shaped or they revealed a slightly curved rod shape (Fig. 6). Given the fact that strain KS, strain BP and strain AG dominate the enrich-

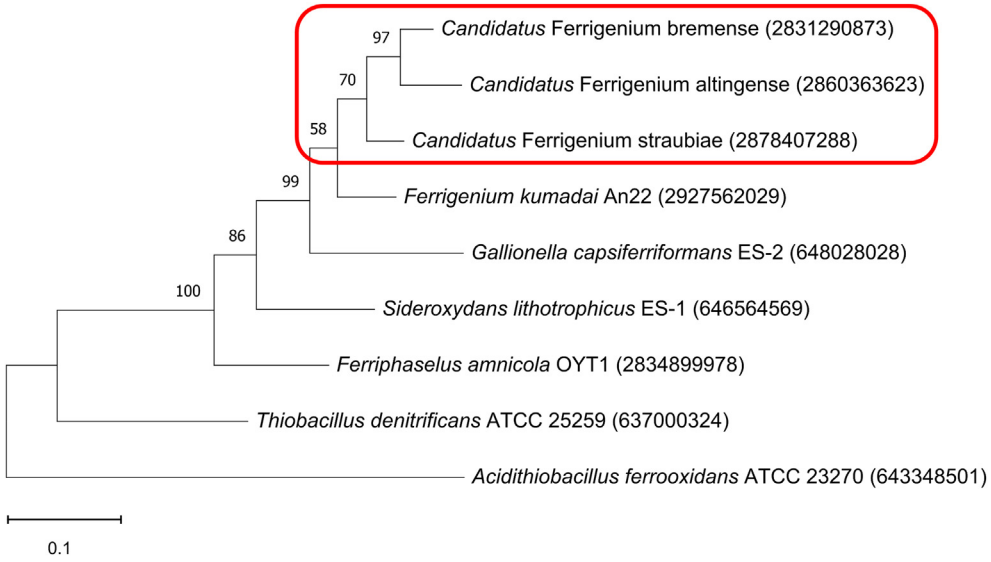

Fig. 4. Phylogenetic tree of nine concatenated house-keeping genes (amino acid sequences) of *rpoA, gyrA, secA, ileS, rho* and *infB* of isolated and *Candidatus* members of the *Gallionellaceae* family and other FeOB, calculated on 1000 replicates using the Maximum Likelihood method. The red box highlights the species classified in this study. The scale bar represents branch lengths measured by the number of substitutions per site. The numbers in brackets show the identifier of each gene in IMG.

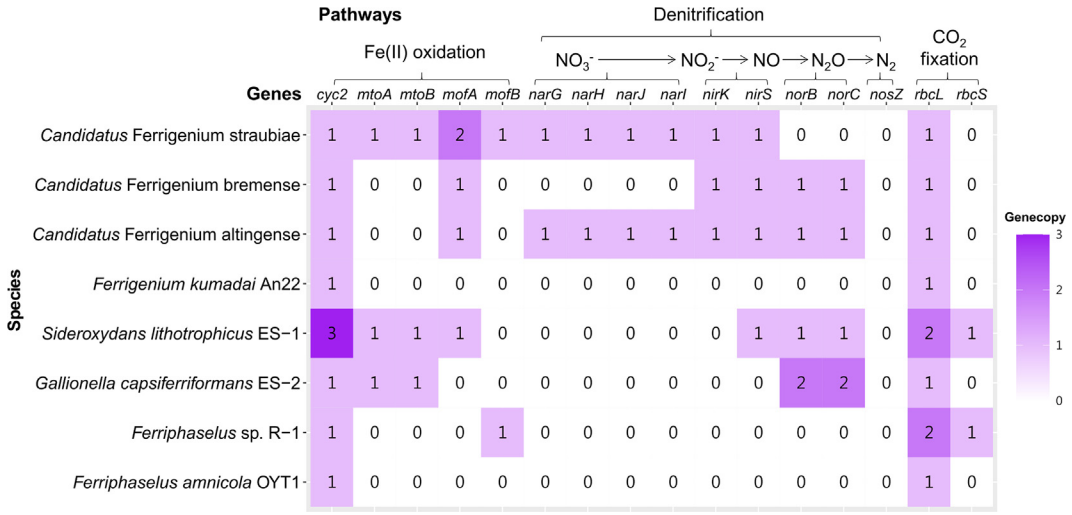

Fig. 5. Summary of key gene copy numbers involved in putative Fe(II)-oxidation, denitrification and carbon fixation of '*Ca.* Ferrigenium straubiae', '*Ca.* Ferrigenium bremense', '*Ca.* Ferrigenium altingense', *Ferrigenium kumadai* An22, *Sideroxydans lithotrophicus* ES-1, *Gallionella capsiferriformans* ES-2, *Ferriphaselus* sp. R-1 and *Ferriphaselus amnicola* OYT1.

ment cultures KS, BP and AG, respectively, we estimated the morphology of strain KS, strain BP and strain AG based on the dominating morphological type of the culture, i.e. the rod or slightly curved rod shaped cells. For culture KS, there were in addition several different imaging analyses performed in previous studies: 4′,6-diami dino-2-phenylindole (DAPI) staining [30,85] and fluorescence *in situ* hybridization (FISH) in combination with fluorescence microscopy as well as helium ion microscopy (HIM) [86]. The results for cell sizes and cell morphology of these imaging analyses corresponded to the SEM observations for strain KS in this study. No diagnostic stalks or capsules were observed for the three cultures using SEM. Most of the cells we observed in all three cultures (i.e. 16 out of 17 cells analyzed for culture KS, 25 out of 27 cells for culture BP and 15 out of 15 cells for culture AG) were closely associated with minerals and in a few cases, cells even seemed to be partly or completely encrusted in Fe minerals, although it remains unknown whether these cells were still alive or whether such encrustation only happened with dead or inactive cells. Additionally, although the genes for flagellar assembly were detected in the three MAGs of strain KS, strain BP and strain AG, the fimbriae and flagella were not observed under SEM. However, during the chemical preparation, the fimbriae and flagella might have been destroyed and, thus, we cannot rule out the existence of fimbriae and flagella. Motility was furthermore not observed for cells of cultures KS, BP and AG using light microscopy.

Environmental occurrence

To estimate the environmental occurrence, i.e. potential niches and preferred habitats, of the three FeOB and closely related organ-

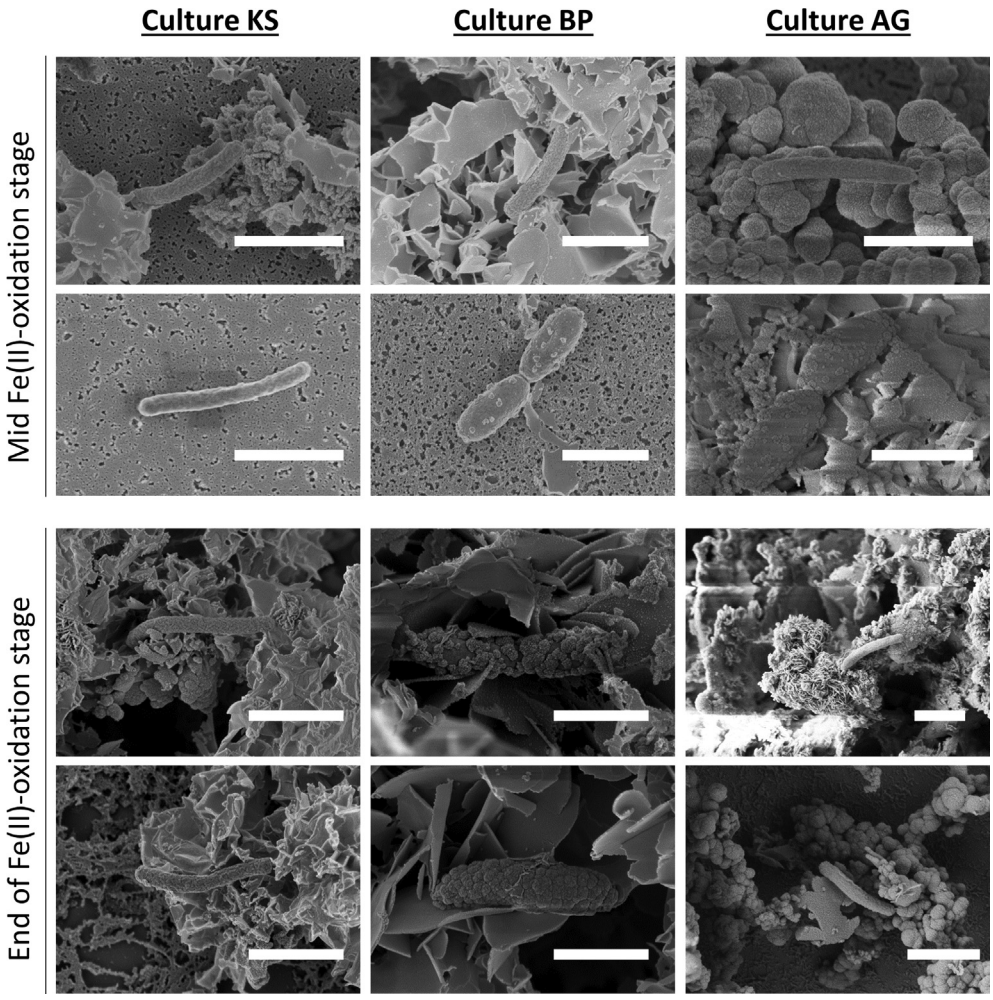

Fig. 6. Scanning electron microscopy (SEM) pictures of culture KS, culture BP and culture AG during the mid Fe(II)-oxidation stage (exponential growth phase) and at the end of the Fe(II)-oxidation stage (lag phase). The scale bars represent 1 μm. Cells were slightly curved rod shaped and partly encrusted with putative Fe(III) minerals in cultures BP and AG (and to a minor extent in culture KS; not shown), especially at end of the Fe(II)-oxidation stage.

isms that affiliate with the same species (and the same strain), nearly full-length 16S rRNA gene sequences that showed a similarity >98.6% (and >99.5%) compared to strain KS, strain BP or strain AG were identified using the BLAST function and the NCBI database (Table S3). For strain KS, the highest similarity (nearly full-length 16S rRNA gene identity 99.52–99.93%) was found for sequences originating from a parallel culture to culture KS cultivated at the University of Wisconsin - Madison and identified as uncultured betaproteobacterium sp. (acc. nos. FN430662.1, FN430666.1, FN430669.1, FN430668.1, FN430670.1, FN430663.1 and FN430659.1; clones F25F63, F29F67, F32F70, F31F69, F33F71, F26F64 and F21F59) [39]. In addition, an uncultured bacterial sequence from an iron-rich microbial mat revealed 99.43% nearly full-length 16S rRNA gene identity (acc. no. LN870688.1; clone Hoffnungsstollen_#4-1A_D09) (Zeitvogel et al., unpublished). For strain BP, the most closely related sequence (nearly full-length 16S rRNA gene identity of 99.26%) was identified as an uncultured bacterium from a polycyclic aromatic hydrocarbon (PAH) degrading bacterial community of a PAH-contaminated soil (acc. no. FQ659636.1; clone I1AB101) [87]. For strain AG, closely related sequences (nearly full-length 16S rRNA gene identity of 100% and 99.93%) were retrieved from a stratified freshwater lake, Lake Mizugaki, in Japan (acc. nos. AB754138.1 and AB754154.1; clones rS43m_43 and rS43m_63) [88]. Additional closely related sequences were identified as an uncultured *Gallionella* sp. (99.93% nearly full-length 16S rRNA gene sequence identity; acc. no. AM167950.1; clone BB03) from spring water consisting mostly of groundwater [89], an uncultured betaproteobacterium sp. (99.80%, 99.53% and 99.53% nearly full-length 16S rRNA gene sequence identity; acc. nos. JQ278897.1, JQ278948.1 and JQ279049.1; clones hmx-114, sf-34 and sz-131) from groundwater [90], and an uncultured bacterium sp. (99.67% nearly full-length 16S rRNA gene sequence identity; acc. no. AY662038.1; clone 015C-C11) from nitric acid-bearing uranium waste contaminated groundwater [91]. The uncultured bacterial sequence from the iron-rich microbial mat (acc. no. LN870688.1; clone Hoffnungsstollen_#4-1A_D09) had 99.79% nearly full-length 16S rRNA gene identity compared to strain AG (Zeitvogel et al., unpublished). From previous studies, it was reported that strain BP has ca. 0.13% relative abundance *in situ* at the origin (i.e. the freshwater pond) [24] and strain AG has ca. 14% relative abundance *in situ* at the origin (i.e. in groundwater samples from the Ammer catchment; Blackwell et al, unpublished), while no *in situ* abundance data were reported for strain KS. In summary, closely related sequences of strain KS, strain BP and strain AG were mainly identified in freshwater ecosystems, e.g. groundwater, pond or ditch sediments, as well as aquifer and wetland soils that probably represent preferred habitats of the three novel FeOB.

Table 2
Phenotypic and genotypic information of '*Candidatus* Ferrigenium straubiae' sp. nov., '*Candidatus* Ferrigenium bremense' sp. nov. and '*Candidatus* Ferrigenium altingense' sp. nov. compared with closely related strains of different genera in the family *Gallionellaceae*.

Characteristics	*Candidatus* Ferrigenium straubiae	*Candidatus* Ferrigenium bremense	*Candidatus* Ferrigenium altingense	*Ferrigenium kumadai* An22	*Sideroxydans lithotrophicus* ES-1	*Gallionella capsiferriformans* ES-2
Isolation source	Sediment	Sediment	Aquifer	Rice paddy soil	Groundwater	Groundwater
Geographic location	Bremen, Germany	Bremen, Germany	Altingen, Germany	Anjo, Japan	Michigan, USA	Michigan, USA
Cell morphology	curved rod	curved rod	curved rod	curved rod	helical rod	curved rod
Cell size (LxW, in μm)	0.8–2.2 × 0.2–0.7†	1.1–2.3 × 0.2–0.6†	0.5–1.8 × 0.2–0.6†	0.9–2.0 × 0.2–0.4	0.3 diameter	0.7 diameter
Stalk formation	N/O	N/O	N/O	-	-	-
Motile/Flagella	N/O	N/O	N/O	+	+	+
Doubling time (h)	9.4 ± 2.9	46.11#	34.98#	6.2	8	12.5
highest relative abundance in the culture	98%	86%	68%	N/A	N/A	N/A
Genome size (bp)	2,659,708	2,446,084	2,180,025	2,572,603	3,003,656	3,162,471
G + C content (mol %)	60.13	58.92	57.97	61.4	57.5	52.8
Contamination	0%	0%	0%	N/A	N/A	N/A
No. of contigs	5	42	9	N/A	N/A	N/A
N50	809,982	97,070	445,488	N/A	N/A	N/A
L50	2	8	2	N/A	N/A	N/A
16S rRNA gene copies	2	1	1	2	2	3
Fe(II) oxidation	+	+	+	+	+	+
Carbon fixation	+	+	+	+	+	+
Growth temperature	28 °C*	28 °C*	25 °C*	12–37 °C	10–35 °C	4–30 °C
Growth pH	6.8–7.2*	6.9–7.2*	6.0–7.0*	5.2–6.8	5.5–7.0	5.5–7.0
Reference	[2,22,23,85]	[24]	[25,29]	[13]	[16]	[16]

N/A: not applicable; N/O: not observed.
†Cell size was estimated using SEM analysis of enrichment culture at a time point where the strain dominated the culture.
#Doubling time of '*Ca.* Ferrigenium bremense' and '*Ca.* Ferrigenium altingense' was estimated by multiplying total cell counts of the enrichment culture obtained by flow cytometry with relative abundance data determined by V4 region 16S rRNA gene amplicon sequencing, respectively, as reported previously [24,25].
*Temperature and pH of the whole enrichment culture KS, BP and AG was measured.

Conclusion

Overall, from the ecological traits, unique metabolic and physiological features, phylogenetic and MAG identity analyses compared to closest related isolated species (Table 2), we propose the name of these three FeOB species as '*Candidatus* Ferrigenium straubiae' sp. nov., '*Candidatus* Ferrigenium bremense' sp. nov. and '*Candidatus* Ferrigenium altingense' sp. nov., named after 'Kristina Straub' who originally isolated culture KS, 'Bremen Pond' the location of culture BP's isolation and 'Altingen Groundwater' the location of culture AG's isolation, respectively. The detailed description of the proposed species is shown in Table 1 and the phenotypic and genotypic information compared to closely related isolates of the proposed species are described in Table 2.

Funding resources

This work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG)-funded research training group [grant number RTG 1708] "Molecular principles of bacterial survival strategies". Natalia Jakus was funded by the Collaborative Research Center 1253 CAMPOS (Project 5: Fractured Aquifers) from the DFG [grant number SFB 1253/1 2017]. Daniel Straub was funded by the Institutional Strategy of the University of Tuebingen (DFG, ZUK63). Nia Blackwell was funded by the Collaborative Research Center 1253 CAMPOS (project 5: fractured aquifers) from the DFG [grant number SFB 1253/1 2017]. Daniel Straub and Andreas Kappler are funded by the cluster of Excellence: EXC 2124: Controlling Microbes to Fight Infections, Tübingen, Germany [project ID 390838134]. Sara Kleindienst is funded by an Emmy-Noether fellowship from the DFG [grant number 326028733].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the infrastructural support by the DFG under Germany's Excellence Strategy, cluster of Excellence EXC2124, project ID 390838134 and the Tuebingen Structural Microscopy Core Facility (funded by the Excellence Strategy of the German Federal and State Governments) for their support and assistance in this work. We also thank the German Research Foundation DFG (INST 37/1027-1 FUGG) for financial support provided for the acquisition of the cryogenic focused ion beam scanning electron microscope. We thank Tillmann Lueders and Zhe Wang for PacBio library preparation and sequencing as well as Bernhard Schink for the recommendation of the nomenclature.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2022.126306.

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