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Repeated horizontal gene transfers triggered parallel evolution of magnetotaxis in two evolutionary divergent lineages of magnetotactic bacteria

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Abstract

Under the same selection pressures, two genetically divergent populations may evolve in parallel towards the same adaptive solutions. Here, we hypothesized that magnetotaxis (i.e. magnetically guided chemotaxis) represents a key adaptation to micro-oxic habitats in aquatic sediments and that its parallel evolution homogenised the phenotypes of two evolutionary divergent clusters of freshwater spirilla. All magnetotactic bacteria affiliated to the *Magnetospirillum* genus (Alphaproteobacteria class) biomineralize the same magnetic particle chains and share highly similar physiological and ultrastructural features. We looked for the processes that could have contributed at shaping such an evolutionary pattern by reconciling species and gene trees using newly sequenced genomes of *Magnetospirillum* related bacteria. We showed that repeated horizontal gene transfers and homologous recombination of entire operons contributed to the parallel evolution of magnetotaxis. We propose that such processes could represent a more parsimonious and rapid solution for adaptation compared to independent and repeated *de novo* mutations, especially in the case of traits as complex as magnetotaxis involving tens of interacting proteins. Besides strengthening the idea about the importance of such a function in micro-oxic habitats, these results reinforce previous observations in experimental evolution suggesting that gene flow could alleviate clonal interference and speed up adaptation under some circumstances.

Introduction

Magnetotaxis couples the biomineralization of ferrimagnetic nanoparticles in organelles called magnetosomes, to a complex system of aero-chemotaxis for guiding the locomotion of magnetotactic bacteria (MTB) parallel to the Earth's magnetic field lines [1]. This function is particularly well described in a group of freshwater MTB of the Alphaproteobacteria class represented by the *Magnetospirillum* genus (MTB_{Mag}). Magnetotaxis is assumed to facilitate vertical navigation towards their optimal niches located at the oxic-anoxic transition zone [2]. Our knowledge of the genetic basis, ecology and biophysical processes involved in magnetite magnetosome biogenesis was built mainly from the study of two model strains, *Magnetospirillum gryphiswaldense* strain MSR-1 [3] and *Magnetospirillum magneticum* strain AMB-1 [4–6]. Since their first observation, numerous other spirilla were isolated and affiliated to the same group based on phenotypic, physiological and morphological features, among which the ability to form magnetosomes [7–11]. As for many prokaryotes, the increasing biodiversity assessment and the development of molecular typing revealed the polyphyletism of magnetotaxis in freshwater Rhodospirillaceae (Alphaproteobacteria) [8, 12]. Members of two genera with different lifestyles: *Phaeospirillum* and *Dechlorospirillum* are actually more closely related to some *Magnetospirillum* lineages than what some *Magnetospirillum* lineages are to each other [7, 8, 12, 13]. The genus *Phaeospirillum* contains spiral-shaped, phototrophic, purple nonsulphur bacterial species [14], while *Dechlorospirillum*, now affiliated to the *Magnetospirillum* genus based on phylogenetic analyses, is represented by non-MTB only [15, 16].

The polyphyletism of magnetotaxis and the lack of a clear correlation between genetic clusters and ecology created some confusion in the classification of these organisms that remains partially resolved [17]. Today, we are still unable to state whether or not magnetotactic *Magnetospirillum* form their own genera [8, 18] because their specific ecological boundaries have not been identified yet and they do not form a monophyletic group [8, 12]. Current data support the existence of two genetically distinct groups

represented by strains MSR-1 and AMB-1 respectively [8]. All magnetotactic strains affiliated to the *Magnetospirillum* genus fix carbon dioxide through the Calvin-Benson-Bassham (CBB) cycle with the presence of a form II ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) gene in their genome [8, 19], and all seem to fix atmospheric nitrogen [20, 21]. Other shared features of described magnetotactic *Magnetospirillum* species relate to biomineralization. They form a single chain of biomineralized cuboctahedral crystals of magnetite, have a bipolarly flagellated helical shape and a respiratory form of metabolism that uses organic acids as a source of carbon and electrons [3]. Moreover, the magnetite synthesis occurs only at very low levels of oxygen or under anaerobic conditions when nitrate is the alternative terminal electron acceptor to oxygen [22–25]. Their metabolic specificities are less obvious to pinpoint, maybe because some traits are variable within species or because they were insufficiently characterized. It seems that their requirement for energy source and carbon source may vary according to the strain. For example, while most species are facultative anaerobes that utilize nitrate as an alternative terminal electron acceptor to oxygen, *M. magnetotacticum* is an obligate microaerophile that requires oxygen even when growing with nitrate [22] and *M. gryphiswaldense* can grow autotrophically using reduced sulphur compounds as electron donor [26].

This high degree of phenotypic homogeneity of magnetic spirilla suggests an adaptation to similar environments that exert important constraints on their metabolism and magnetotaxis. From an evolutionary perspective, the common genetic determinism to all MTB indicates a single emergence of magnetotaxis in prokaryotes. Magnetosome biogenesis is encoded by unique genes (referred as the *mam* and/or *mms* genes) that are, for most of them, clustered into operons within a specific genomic region [27–29]. Compiling studies show both evidence of vertical inheritance of these genes [12, 30, 31] and multiple acquisitions by horizontal gene transfer (HGT) in some lineages in MTB of the Alphaproteobacteria class [32–34] with duplication events [34, 35]. These observations raise questions on the evolutionary mechanisms shaping the adaptation to this ecological niche

and the phenotypic maintenance at such level of phylogenetic distance between two evolutionarily distinct lineages.

Selection contributes to divergent evolution and different ecological speciation in the presence of reproductive isolation [36]. However, similar environments in different locations may promote parallel or convergent evolution of traits and maintain a high degree of phenotypic similarity in independent and divergent populations [37]. Tracking the genetic parallelism involved in parallel ecological speciation may help to better understand the mechanisms of adaptive evolution. Because similar niches may exert the same degree of evolutionary constraint on traits, the same *de novo* beneficial mutations are likely to be repeatedly selected and fixed in populations [37–39]. In microorganisms, other evolutionary forces than mutation, like homologous recombination or orthologous replacement mediated by HGT generate genetic variation [40–42]. In prokaryotes, the contribution of these mechanisms in parallel evolution specifically has been less documented [43], although HGT and homologous recombination could promote the repeated exchange of beneficial alleles in genetically distant organisms, purge the genic variability to maintain phenotypic and ecological cohesiveness in an ecotype [44]. In laboratory-controlled experiments, recombination has been shown to alleviate clonal interference and accelerate adaptation of *Escherichia coli* populations under some circumstances [45]. *In natura*, two divergent populations inhabiting the same habitat are more likely to exhibit weaker barrier to gene flow than do different ecotypes, regardless the genetic distance [42, 46]. Our understanding of these processes leading to ecological differentiation in bacteria suffers from the lack of niches and habitats studied. Indeed, our vision has been built mainly from studies on pathogens [44]. But a broader investigation of environmental niches and ecotypes may help to map bacterial diversity onto ecology involving other traits than those related to cell surface, DNA binding and pathogenicity-related functions [47].

Here, we investigated the evolutionary mechanisms involved in the parallel evolution of magnetotaxis in two distinct groups of magnetotactic *Magnetospirillum* species. We particularly tested the hypothesis that recombination *sensus lato* could be involved in the maintenance of highly homogenous phenotypes in two divergent lineages

keeping them adapted to very similar niches. We studied the biology and genome of newly isolated freshwater magnetotactic and non-magnetotactic *Magnetospirillum* related species. By comparing gene contents and inferring phylogenies based on whole genomes and magnetosomes associated genes, we showed that ancestors of these groups exchanged their magnetosome gene clusters several times over the course of magnetotactic *Magnetospirillum* diversification, which might have been at the origin of the maintenance of a high degree of global genomic conservation and phenotypic similarity. Our results showed that repeated gene exchanges of entire operons involved in sensing and motility may contribute to the parallel evolution of species facing similar environmental constraints, which likely accelerate their adaptation to microoxic conditions.

Materials and methods

Genomes collection and growth of bacteria

Draft and closed genomes of all Rhodospirillaceae available in October 2017 were downloaded from the public repository database at NCBI and uploaded into the MicroScope platform for their automatic and manual annotation [48]. Sequencing and assembling of *Magnetospirillum magneticum* AMB-1 [49], *M. gryphiswaldense* MSR-1 [50], *M. magnetotacticum* MS-1 [51], *M. caucaseum* SO-1 [52], *M. marisnigri* SP-1, *M. aberrantis* SpK [53], *M. bellicus* VDY [54], *Magnetospirillum* sp. XM-1 [55], *Magnetospirillum* sp. ME-1 [56], *P. fulvum* MGU-K5 [57], and *P. molischianum* DSM120 [58] were described previously. Draft genome assemblies of non-magnetotactic *Magnetospirillum* strains CP2, CP3, VDY and WD, and those of *Phaeospirillum* strains DSM 114, DSM 115, DSM 117 and DSM 13234 were provided by the Joint Genome Institute (www.jgi.doe.gov) and described in supplementary **Table S1**. This list was completed with 5 genomes sequenced in this study. Genomes of magnetotactic strains LM-1, SS-4, UT-4, LM-5, and CP-1 were sequenced and assembled following the exact procedure described in [34] based on genomic DNA obtained from axenic cultures. Their sampling sites, the isolation procedure and culture medium were described in [8], with the exception of strain CP-1 reported here for the first time. Strain CP-1 was isolated using a similar procedure as previously described [8], from the freshwater river Couze Pavin, Auvergne, France (45°30'35.0"N, 2°54'12.6"E). Chemolithoautotrophic growth experiments were carried out on strains AMB-1, SS-4, CP-1, MSR-1, UT-4, LM-1 and LM-5 using a similar semi-solid growth medium as previously described [8] but removing sodium acetate and sodium sulphide and adding after autoclave 3 ml of 40 % sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$). Thiosulfate was deemed positive for the support of growth if three conditions were met: (i) a band of cells formed in the tube, (ii) the band after growth was significantly thicker than the control containing no thiosulfate and (iii) cells continued to grow in the same medium in three successive transfers. The draft genome sequences partially annotated of LM-1, SS-4, UT-4, LM-5, and CP-1 were

170 submitted to the European Nucleotide Archive and carry the accession numbers
171 PRJEB35448, PRJEB35447, PRJEB35446, PRJEB35445 and PRJEB35444, respectively.

172 **Light and electron microscopy**

173 Motility and magnetotactic behaviour of the different *Magnetospirillum* strains were
174 analysed and recorded under the light microscope Leica LMD6000 equipped with the
175 camera Leica DMC 4500. Transmission electron microscopes (TEM) was used to analyse the
176 ultrastructure of *Magnetospirillum* strains directly deposited onto TEM copper grids coated
177 with a carbon film. Magnetosomes shape and organization were visualized with a Tecnai
178 G2 BioTWIN (FEI Company, Eindhoven, Netherlands) equipped with a CCD camera
179 (Megaview III, Olympus Soft imaging Solutions GmbH, Münster, Germany) with an
180 accelerating voltage of 100 kV.

181 **Comparative genomics**

182 Evolutionary relationships between *Magnetospirillum*-related species and their closest
183 members of the Rhodospirillaceae family were investigated using whole genome sequences.
184 Clusters of orthologous were defined using the OrthoMCL clustering algorithm
185 implemented in GET_HOMOLOGUES open-source software [59]. We considered any
186 cluster of orthologous proteins for which pairwise BLASTP alignments had an expectation
187 value $E < 10^{-5}$ and a minimal protein coverage of 50%.

188 Genomes and encoded proteomes of *Magnetospirillum* species and their closest relatives
189 were compared with several complementary algorithms to define sub-groups/species based
190 on their nucleotidic/amino acid composition : (i) Average Nucleotide Identity (ANI) and
191 Average Amino acid Identity (AAI) values were calculated using the ANI/AAI-Matrix
192 online service (http://enve-omics.ce.gatech.edu/g-matrix/) [60] using reciprocal best hits
193 (http://enve-omics.ce.gatech.edu/), (ii) digital DNA-DNA hybridization (dDDH) values
194 were determined using the Genome-to-Genome Distance Calculator (GGDC 2.1;
195 https://ggdc-test.dsmz.de/ggdc.php) using the recommended BLAST+ alignment and

196 formula 2 (identities/HSP length) [61] and (iii), percentages of conserved proteins (POCP)
197 [62] were calculated using the script runPOCP.sh [63].

198 Metabolic pathways were predicted with the Microscope platform [64] using the Pathologic
199 algorithm [65], which computes an initial set of pathways by comparing genome
200 annotations to a collection of microbial Pathway/Genome Databases (PGDBs) MicroCyc
201 derived from the metabolic reference database MetaCyc [66]. For each MicroCyc pathway,
202 the tool gives the completion value referring to the number of reactions in a given
203 strain/total number of reactions in the same pathway defined in the MetaCyc database.

204 The presence of conjugative system was checked in all genomes using MacSyFinder
205 software and the TXSScan module [67, 68], which uses HMM profiles for efficient genomic
206 detection of bacterial secretion systems encountered in diderm-LPS bacteria, and their
207 discrimination from homologous machineries.

208 **Statistical analyses**

209 Statistical analyses were performed in R version 3.5.1 [69]. Averages of AAI and POCP
210 values estimated from the pairwise comparison of genomes sets were compared either by
211 the Student's t-test or by the non-parametric Mann–Whitney U test (if the underlying
212 assumptions of the first test were not satisfied). Differences were considered significant
213 when P-values were below 0.05.

214 A principal component analysis (PCA) was performed using the ade4 package [70] to
215 compare metabolic pathways of the 23 *Magnetospirillum* relatives using a reduced matrix
216 of MicroCyc pathway completion values generated with the MicroScope platform. The
217 reduced matrix consisted of the 99 pathways whose completion was variable between
218 groups among the 410 non-redundant pathways detected in at least one genome.

219 **Phylogenetic analyses**

220 For phylogenetic analyses, only clusters with single-copy sequences were taken into
221 consideration and in-paralogs were excluded. All complete gene outliers were removed

222 with Phylo-MCOA software [71]. For the species tree, amino acids sequences of 839
223 orthologous proteins were aligned independently using MAFFT [72] and alignments were
224 trimmed using BMGE [73] setting block length to 3, and concatenated into a final alignment
225 of 269,968 amino acids positions among which 151,377 were polymorphic. The maximum
226 likelihood tree was built from the concatenated sequences with IQ-TREE [74] using a
227 partition model; the substitution model for each protein was selected by ModelFinder [75]
228 with the Bayesian Information Criterion (BIC). The statistical support of the branches was
229 estimated by the ultrafast bootstrapping approach implemented in IQ-TREE applying 1000
230 replicates. A previous study suggesting that strain LM-1 was a new freshwater MTB*Mag*
231 genus [8], we included this strain in the external group for phylogenetics and comparative
232 genomics.

233 Evolution of genes encoding for proteins involved in magnetosome biogenesis was studied
234 by reconciling binary gene trees built from gene-by-gene alignments of *mam* genes or their
235 concatenation with the non-binary species tree under the DTL parsimony algorithm
236 implemented in Notung 2.9 software [76]. The algorithm captures gene duplication (D),
237 transfer (T) and loss (L) driving tree incongruence and infers all optimal solutions to finally
238 report the complete and temporally feasible event histories giving the data.

239

240 ## Results

241 ### Species of magnetotactic *Magnetospirillum* do not form a monophyletic group at the whole
242 ### genome level

243 We built a phylogenetic framework to study the evolution of magnetotaxis in MTB$_{Mag}$ at
244 the whole genome scale using draft genomes of freshwater magnetotactic and non-
245 magnetotactic bacteria close to *Magnetospirillum gryphiswaldense* MSR-1 or *M.*
246 *magneticum* AMB-1 (Table S1). A first phylogenetic tree based on all proteins shared by
247 strains representing the Rhodospirillaceae diversity and related other Alphaproteobacteria
248 confirmed that all 23 *Magnetospirillum* relatives in this study cluster together at the
249 genome level and positioned strain LM-1 as the closest freshwater strain of this group (Fig.
250 S1). Based on this phylogeny and what we currently know about the biology of strain LM-
251 1 [8], this strain represents a new genus. Whereas *Magnetospirillum* species exhibit a
252 spirillum shape with flagella at both poles and synthesize cuboctahedral crystals, strain LM-
253 1 displays a vibrio shape with a single polar flagellum and synthesizes elongated prismatic
254 crystals resembling those of *Magnetovibrio blakemorei* strain MV-1 [8]. Despite these
255 morphological similarities between MV-1 and LM-1, the proposed LM-1 genus is much
256 closer to freshwater Rhodospirillaceae MTB than to marine Rhodospirillaceae MTB. Thus,
257 we propose the name *Candidatus* Magneticavibrio boulderlitore (the magnetic vibrio
258 isolated from the shore of boulder beach in Lake Mead) [8]. We further used strain LM-1
259 as a control and external outgroup to *Magnetospirillum* related taxa in this study.

260 The phylogeny of *Magnetospirillum* related species only was then reconstructed based on
261 839 orthologous proteins selected after all complete gene outliers were removed **[71]**. The
262 tree topology was partially congruent with those of the trees built from 16Sr RNA sequences
263 of some strains in previous studies [8, 17]. For example here, the strain UT-4 clusters with
264 the MSR-1 clade (Fig. 1A), while it was supposed to be ancestral to all MTB$_{Mag}$. The strain
265 CP-1, isolated here from the Couze Pavin River, France, clusters with the ancestor of all
266 AMB-1 related strains. Together, all *Magnetospirillum* related species form four

monophyletic groups (namely SpK, MSR-1, MGU-K5 and AMB-1 clades), among which two: MSR-1 and AMB-1, are composed of magnetotactic bacteria only. The non-magnetotactic MGU-K5 clade (*Phaeospirillum* species) shares a more recent ancestor with the AMB-1 clade composed of strains ME-1, XM-1, SO-1, AMB-1, SS-4, SP-1 and CP-1. The SpK clade (formerly *Dechlorospirillum* including *M. aberrantis*) is composed of non-magnetotactic bacteria only (CP2, CP3, VDY, WD and SpK) and shares a more recent ancestor with the MSR-1 clade composed of magnetotactic strains LM-5, BB-1, MSR-1 and UT-4. These two polyphyletic groups regarding magnetotaxis relate to each other with a direct common ancestor.

Lifestyle and environment maintain genetic relatedness and phenotypic homogeneity in two divergent magnetotactic lineages

The intriguing polyphyletism of magnetotaxis in MTB*Mag* and the high degree of phenotypic divergence of species of the MGU-K5 and SpK clades raised questions about the definition of *Magnetospirillum* as a genus. By calculating several indexes that are generally used to refine taxonomic boundaries for prokaryotes like the AAI, ANI, dDDH or POCP, we investigated the support of their classification at the genome level (Fig. S2 to S5). The comparison of genomes pairwise within and between monophyletic groups confirmed the existence of four clades for which AAI values ranged from 64% to 70% (Fig. 1c). These values justify a new genus description for each group according to previous large metadata analyses assuming a significant ecological differentiation [60]. Here, it was interesting to observe higher POCP values between all magnetotactic species than between these species and their genetically closer non-magnetotactic species, which showed that MTB lifestyle fostered the maintenance of similar genome contents (Fig. 1c). On the basis of their phenotype, different magnetotactic species studied here and previously were difficult to distinguish using light and electron microscopy approaches [3, 4, 7–11, 26, 77]; they share all very similar magnetotactic behaviours, motilities, cell ultrastructures and magnetosome morphology/organization (Fig. S6). Experimental attempts to find a different physiology specific to one of the two groups failed too. According to the literature, chemolithoautophy

with thiosulfate as electron donor was potentially the only difference between AMB-1 and MSR-1 [26].

We tested this hypothesis *in silico* by comparing whole genomes and noticed that some genes involved in the thiosulfate-oxidizing Sox enzyme system were specific to the MSR-1 clade (Table S2). Testing chemolithoautotrophy with thiosulfate as electron donor for strains of both clades, we showed that not all members of the MSR-1 group, such as strain LM-5, were able to use thiosulfate as energy source, while none of the cultured species tested of the AMB-1 group grow in such conditions. No other clear interpretation could be made about the biological differences between magnetotactic *Magnetospirillum* lineages. We thus looked for other genomic evidences of clear biological boundaries by reconstructing the metabolic pathways. Principal component analysis (PCA) built from the matrix of pathway complementation (Fig. 1d, Table S3), confirmed that *Phaeospirillum* species do not cluster with the other lineages and that AMB-1 clade is metabolically closer to that of MSR-1, than MGU-K5 and SpK clades. Searching pathways that participate the most to the variability for both PCA axis, we confirmed that MGU-K5 relatives use light-harvesting complexes with the impossibility to denitrify, degrade alanine or produce vitamin B_{12}. Differences between the three other groups were much less significant and were associated, for example, to creatinine degradation, cyclopropane fatty acids, spermidine and preQ0 metabolite biosynthesis. In the absence of significant ecological differentiation for the non-photosynthetic clades, there is no support for the creation of two genera for AMB-1 and MSR-1 clades.

Conservation of *mam* genes composition and synteny supports magnetotaxis was functionally constrained by similar selective pressures

Draft versions of the magnetosome gene clusters (MGCs) were reconstructed for the new genomes, and despite the incompleteness of some operons, several observations were validated. First, the *feoABm*, *mms6*, *mamGFDC*, *mamAB* and *mamXY* gene clusters encoding proteins involved in magnetosome biogenesis were found in all genomes with a very high degree of synteny conservation within operons (Fig. 2). Most of the strains carry

an additional *mamJOE-like* gene cluster downstream *mamV* and *mamW*. If present, this cluster of paralogs was followed by several orthologous proteins localized upstream *mamXY* operon, among which some are specific to MTB$_{Mag}$. For instance, an homolog of the ferric uptake regulation protein Fur is often present in the magnetosomes gene cluster and in *M. gryphiswaldense* MSR-1, an homolog that was previously shown to affect magnetite biomineralization, is present outside the MGC **[78].** With the exception of LM-5, whose MGC organization is almost identical to that of the AMB-1 cluster, strains of the MSR-1 group harbour a slightly different structure. The apparent global conservation of the MGC in *Magnetospirillum* spp. could support their orthology in freshwater MTB$_{Mag}$. However, we found several genomic features, typical of mobile regions and genomic islands that could be responsible for genomic instability, recombination and HGT. Transposases, integrases and recombinases (e.g. homologs to y4qJ, members of the IS110/IS3/IS407 family) were found in the vicinity or within the MGCs of several strains (e.g. AMB-1, ME-1, UT-4, MSR-1), some of which are phage-related or even involved in a functional conjugation system (e.g. *virB6*, *virB8*, *virB9* of type T Type 4 Secretion System) **[79, 80]**. It should be noted the presence of two copies of *mamJ* in the *mamAB* operon of strain LM-1. Although the percentage of similarity of its encoded protein is low compared to MamJ copies of members of the *Magnetospirillum* genus, it shows that this gene is not restricted to the magnetotactic spirilla from freshwater. In strains MSR-1 and AMB-1, MamJ was shown to interact with MamK filament and thus involved in magnetosome chain formation **[81, 82]**.

Operons involved in magnetotaxis were acquired and lost repeatedly in the course of the *Magnetospirillum* evolution

The evolutionary history of magnetotaxis was investigated by reconstructing phylogenies based on known magnetosome-associated proteins described in MSR-1 and AMB-1 genomes that are shared by all strains used in this study. We checked the automatic annotation manually gene by gene for rare ambiguous cases. Gene orthology was then tested by comparing gene and species trees topologies. Trees reconstructed from each of the 31 *mam* genes/proteins conserved in all *Magnetospirillum* draft and closed genomes were

consistent with each other within putative operons. However, some nodes were not always well statistically supported because some amino acids (AA) and DNA sequences were almost identical between genomes. Indeed, for numerous magnetosome-associated proteins, AA sequences identity percentage was up to 25% higher than the average AAI value. For example, MamH sequences were 100% identical between AMB-1, ME-1 and SP-1 while the AAI values of their genomes compared pairwise were between 75 and 87%. Unless a strong negative selection pressure purified polymorphism over *Magnetospirillum* speciation history, such result was congruent with allelic exchange. Trees topologies built from the concatenation of Mam proteins and genes were incongruent with that of the species evolutionary history (Fig. 3). They showed magnetotaxis has a common origin, but does not share the same ancestry in *Magnetospirillum* and has been regularly transferred between ancestors by homologous recombination and/or HGT events. The *mms6*/*mamGFDC* and *mamAB* operons had the same history (Fig. S7) so data were combined to build one tree (Fig. 3b). This history was different for the *mamXY* operon (including here the *mag* genes) (Fig. 3c). The *feoAB* evolution could not be totally resolved because the polymorphism was too low and the degree of site saturation too high (Fig. 3d). From these trees, the most obvious incongruences between the species tree and magnetotaxis trees were for the relationships between strains LM-5 and CP-1 with the rest of the lineages, even if conflicting signals were globally observed for all strains. Indeed, we confirmed at the whole genome level that although strain LM-5 belongs to the MSR-1 group, its magnetosome genes were most closely related to species of the AMB-1 group [8]. For strain CP-1, isolated in this study, it is the opposite; CP-1 belongs to the AMB-1 group but its MGC shared a more recent ancestry with bacteria of the MSR-1 group.

To go further, we inferred the most parsimonious scenarios explaining such evolutionary histories of the magnetosome genes by reconciling these gene trees and the species tree under the duplication-transfer-loss (DTL) model implemented in Notung 2.9 software [83] (Fig. 4). Under such a model, the ancestor of the four monophyletic groups of magnetotactic and non-magnetotactic species had a magnetotactic ancestor. Magnetotaxis was then lost in the SpK and MGU-K5 groups (Fig. 4 and Fig. S8). The MGCs of the UT-4

and CP-1 strains represent the most ancestral forms of the MGC in the MSR-1 group and AMB-1 group, respectively. However, an ancient HGT event replaced the whole MGC of the most recent MSR-1 group species by an ancestral form that has emerged anciently first in the AMB-1 clade. This event was followed by other similar events of orthologs replacement during the *Magnetospirillum* diversification within and between groups. Importantly, several paralogs of the *mamQ*, *mamR* and *mamB* genes seem to have been horizontally transferred together with the *mamAB* operon during the AMB-1 clade diversification. These genes are identical or nearly identical in the closest lineages of the AMB-1 clade and have likely emerged recently. Further analyses using a larger dataset will be needed to test the evolutionary relationships of these paralogs with the *mamJOE*-like gene cluster. This later likely emerged from duplication of *mamJOE* in the ancestor of all *Magnetospirillum* spp. of AMB-1 group (Fig. 4). The *mamQRB* paralogs present in AMB-1 closest relatives seem to have emerged from a different and more recent duplication event than that linking the *mamMOBQ* paralogs in CP-1, SP-1 and SS-4.

Discussion

Determining the evolutionary forces shaping genomic polymorphism allows to identify key features involved in microbial adaptation and to resolve their evolutionary histories. In prokaryotes, genetic parallelism was identified by the detection of repeated emergences of the same *de novo* beneficial mutations [37–39]. Here, we showed that multiple allelic exchanges of whole operons mediated the parallel evolution of magnetotaxis in two evolutionarily distinct groups of bacteria. We thus provide evidence that another force than mutation can participate in the maintenance of highly similar phenotypes within a group of phylogenetically distant prokaryotes sharing the same ecological niche. Parallel evolution of distantly related populations is favoured when they experience similar selection pressures [37, 43]. Freshwater magnetotactic species cluster into two genomic genera for which it was difficult, even impossible, to identify biological and ecological boundaries. Although, these two species clades represented by AMB-1 and MSR-1 strains, could represent two different genera and carry on different names, we thus believe that the absence of these boundaries impedes the amending of the *Magnetospirillum* genus name of either the AMB-1 or MSR-1 groups.

Here, these results raise several questions about the role of magnetotaxis with microbial adaptation to habitats at the oxic-anoxic interfaces of freshwater sediments. A part of MTB$_{Mag}$ similarity could not be associated to phylogenetic inertia solely, but more likely to adaptation of similar environmental constraints [84]. Did magnetotaxis trigger the ecological cohesiveness of these divergent lineages? Indeed, magnetotaxis is known to optimize bacterial motility along with redox and chemical gradients [2, 85]. Repeated acquisition of beneficial alleles could thus speed up microbial adaptation to microoxic settings by optimizing the sensing or facilitating magnetosome formation in specific conditions. Instead, it is also possible that these narrow and unstable niches selected few specialized catabolic reactions on which magnetosome biogenesis depends on [86–89]. In that case, magnetotaxis selection would arise secondarily from divergent populations already ecologically cohesive that exploit the same redox reactions and carbon sources. According to the theory, this cohesiveness arises when few adaptive solutions exist to face

the environment, which in turn fosters the genetic parallelism or even convergence [37]. Gene flow and allelic exchange are known to allow the recipient bacteria with pre-existing adaptations to invade a new niche. However, in this specific situation, it can also improve its performance in its current niche [90]. Given our data, this second scenario is the most parsimonious explanation, but in any case, they both support that magnetotaxis is an important adaptation to oxic-anoxic interfaces.

For years, studies compiled evidences of the MGC transferability between MTB and non-MTB. Adaptive transfer of genes is limited to those that can be transferred as a functional unit, provide a niche-transcending adaptation, and are compatible with the architecture and physiology of other organisms [42]. To make the exchange possible, genes must fit on a chromosomal segment short enough to be transferred or they must fit on a mobile extrachromosomal element [41]. The MGC totally fits with all these requirements. First, the heterologous expression of *mam* operons in foreign species with compatible physiology led to the formation of magnetosomes [91]. Then, these genes are surrounded by mobile elements from type IV secretion systems involved in conjugation, transposases with a high degree of conservation and phage elements [29, 92]. Interestingly, numerous similar mobile elements are surrounding the magnetosome islet, observed so far in the genome of *M. Magneticum* AMB-1 only [93, 94]. This genomic region harbours few *mam* genes homologs, whose acquisition has been related to an HGT event from the *Ca.* Etaproteobacteria strain MC-1. However, this scenario is unlikely because of their homology with *Magnetospirillum mam* genes. Data rather support that the AMB-1 islet is the remnant of one or several independent ancient events (including duplication and/or HGT) that occurred in the early time of *Magnetospirillum* groups emergence. Currently, the lack of sequence conservation and the dramatic evolutionary acceleration of Mam homologs in the islet could result from a neo-functionalization and prevent to fully resolve their history in the light of the current data.

So far, the contribution of gene flow and allelic exchange to magnetotaxis evolution was formally evidenced for ancestors of closely related bacterial genera only [34]. The current evolutionary scenario proposes that these forces did not contribute to magnetotaxis

453 evolution at larger taxonomic scales [30, 31]. However, this unexpected scenario relies on
454 big assumptions, which led to other authors to question it and to state that HGT contributed
455 in the early steps of phyla emergences [33]. Only new genomes of deep branching lineages
456 and proper analyses will resolve MTB evolution that very likely involve mutation,
457 recombination and duplication events. The parallel evolution of magnetotaxis observed in
458 the *Magnetospirillum* clades may have occurred at larger taxonomic scales between MTB
459 donors and non-magnetotactic recipient lineages with compatible physiologies and sharing
460 the same ecological niches. The successful transformation of *Rhodospirillum rubrum* [91]
461 with the MSR-1 MGC showed that such event is possible. In most aquatic habitats, oxic-
462 anoxic interface niche represents a tiny layer of ~100 µm in thickness where
463 microaerophilic microorganisms thrive due to the overlap of reduced and oxidized chemical
464 species. This layer is a very coveted zone and thus a hot spot for microorganismal adaptation
465 where adaptive solutions may be reduced [95].

466 Multiple genome sequencing projects revealed years after years the multi-faceted
467 evolution of magnetotaxis with evidences of both vertical and horizontal inheritance [17,
468 31–34]. Despite the polyphyletism of magnetotaxis at the phylum level, some authors
469 argued for a vertical inheritance of the MGC followed by multiple and independent losses
470 in non-MTB clades. Here, we showed further evidence that genetic exchanges may explain
471 this polyphyletism at the genus level.. Because our sample was limited to few strains
472 collected worldwide over a large time scale, we undoubtedly underestimated the number
473 of events, including potential duplications [35]. Repeated *de novo* mutation might have also
474 contributed in some extent to the parallel evolution of the function, but our approach was
475 not suitable to identify such events. In the case of magnetotaxis, recombination between
476 distantly related bacteria could represent a more parsimonious and rapid solution for
477 adaptation compared to independent and repeated *de novo* mutations. The reason comes
478 from the magnetosome gene clusters architecture: magnetosome biogenesis relies on a set
479 of operons involving tens of interacting proteins that certainly coevolved to optimize the
480 function [5]. Thus, any mutation optimizing the function of a single gene would not
481 necessarily optimize the function itself and could even decrease it if other beneficial

mutations do not occur at the same time. Selection acting at the operon level rather than on genes individually, homologous recombination of entire operons could rapidly purge the diversity and spread the beneficial mutations in the population. It is important to further test these hypotheses using MTB$_{Mag}$ and experimental evolution approaches. Such study would definitively quantify the relative role of gene flow compared to mutation in speeding up adaptation when this later involves large operons of interacting genes and functions as complex as biomineralization.

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Conflicts of interest

The authors declare no conflict of interest.

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764

765 ## Figure legends

766 **Fig. 1** Genomic insight into the evolution and similarities of *Magnetospirillum* relatives. **a**
767 Phylogeny of *Magnetospirillum* species rooted with strains representing other
768 Rhodospirillaceae species (grey). The Maximum-likelihood tree was drawn to scale and
769 branches length represents the number of base substitutions per site. Black circles represent
770 nodes supported by 100% of the replicates. Names in bold represent MTB strains. Strains
771 related by branches with the same colour represented different clades distinguished here,
772 and could be considered as different genera according to the average amino-acid identity
773 (AAI) (Fig. S2) and their lifestyle. **b** Histograms drawn to scale showing the number of
774 orthologous proteins specific to a group of bacteria that are shared by all members of this
775 group (black bars represent 100 proteins). The first, second and third histograms give the
776 number of proteins specific to (1) each clade, (2) the MSR-1/SpK group vs. AMB-
777 1/*Phaeospirillum* group, and (3) MTB groups vs. non-MTB, respectively. **c** Pairwise
778 comparison of percentages of conserved proteins (POCP) (Fig. S3) and average amino-acid
779 identities (AAI) between the strains of the four groups ordered according to the
780 phylogenetic tree presented in panel **a**. Circles and colours are proportional to the values
781 that represent averages and standard errors calculated from the pairwise comparisons
782 within each group. All the values are significantly different from each other (Student t-test,
783 n=23, $P < 0.05$). **d** Principal component analysis of the metabolic networks of strains
784 predicted with the MicroCyc resource implemented in MicroScope with the projection of
785 ellipses and gravity centres of classes representing the phylogenetic groups.

786 **Fig. 2** Composition and gene synteny of the *feoABm*, *mms6*, *mamGFDC*, *mamAB* and
787 *mamXY* operons encoding for proteins involved in magnetosome biogenesis in
788 magnetotactic *Magnetospirillum* species and other conserved genes and *mam* paralogs with
789 putative function in magnetosome biogenesis. Sequences are organized according to species
790 phylogeny. Homologous genes are symbolized by the same letters and their relative 5'-3'
791 orientation compared to each other's is given by the bottom line colour (red or black). A
792 vertical blue dotted line between two genes symbolizes a truncation (contig edge) while a

grey slash denotes that two genes are related through a continuous genomic region of one or more genes. Genes are next to each other if they are not separated by a slash. The additional paralogous *mam* genes (the *mamJOE-like* operon or a duplicated genes set of *mms6*, *mmsF*, *mag1* and *mag3* for example) are associated with a quotation mark. Paralogous *mam* genes were found on very short contigs outside the MGC in CP-1 and were not shown on this figure (*mamOBQ* and *feoABm* for example). Genes *h1* to *h4, m1* to *m3* and fur_m represent unknown homologous genes conserved in many MGCs, the *mag* genes previously described [34] and an homolog of the ferric uptake regulator *fur* specific to the MGC, respectively. The accession number of these genes in the reference genome AMB-1 are BAE49759, BAE49764, BAE49812, BAE49814, BAE49823, BAE49824, BAE49825 and BAE4981, respectively.

Fig. 3 Evolutionary history of proteins involved in magnetotaxis in *Magnetospirillum* relatives. **a** Species tree from Figure 1a. Gene trees built from the concatenation of Mam proteins present in **b** *mms6*, *mamGFDC* and *mamAB* operons, **c** the *mamXY* operon (including here the *mag1, 2* and *3* genes) and **d** the *feoABm* operon. Strains names and external branches are coloured according to the sub-groups of the first panel to facilitate the visualisation of relationships in gene trees. Gene trees were rooted with the other magnetotactic Alphaproteobacteria of the Figure 1a including strain LM-1.

Fig. 4 Most parsimonious scenario describing the evolutionary history of *mms6*, *mamGFDC* and *mamAB* operons along with the diversification of *Magnetospirillum* species. Events of gene duplication (D), loss (L) and transfer (T) were inferred using the duplication-transfer-loss (DTL) model of Notung 2.9 software **[76]** by reconciling the tree based on the concatenation of shared *mam* AA sequences (thin cladogram, Fig. 2b) with the species tree (thick grey cladogram, Fig. 1). Colours symbolize divergent clades or lineages of the species tree. The colour of the internal branches show the ancestry of the genes encoding for magnetosome-associated proteins. For horizontal gene transfer events, the name of the donor lineage associated is given. The black solid lines represent the vertical inheritance of the genes while the black dotted lines represent their loss. D* denotes a putative duplication

821 event that could have occurred in the ancestor of AMB-1 clade and led to the emergence of
822 both ancestors of the *mamJOE-like* cluster.