enrichment cultures down to a depth of 500 m in Black Sea cultures, approximately 400 m below the suboxic-anoxic interface. Although Jørgensen *et al.* (*27*) found that $S_2O_3^{2-}$-oxidizing bacteria composed 0.01 to 0.6% of the total bacterial numbers near the interface (10^1 to 10^3 cells per milliliter), *Shewanella* species made up 20 to 50% of the total bacterial counts in the suboxic zone (10^5 cells per milliliter) (*29*). Therefore, the lack of HS^- oxidation products in the Black Sea and in other environments where SO_4^{2-} reducers are absent may be due largely to the presence of *S. putrefaciens*. This microorganism may also be important where SO_4^{2-} reducers are present because of its ability to utilize a wider range of carbon substrates (*2*, *3*).

The manipulation of sulfur species by *S. putrefaciens* can be summarized by reactions 5 to 7 in Table 1. The respiratory versatility of *S. putrefaciens* may give it a distinct metabolic advantage in suboxic and anoxic environments such as reducing sediments or stratified basins like the Black Sea, where the concentrations of various electron acceptors change with time and depth. This microorganism may play an important role in both trace metal and sulfur cycling between oxic and anoxic environments. Although *S. putrefaciens* readily reduces solid-phase iron and manganese, it also utilizes the more soluble oxidation products of HS^- (for example, $S_2O_3^{2-}$), released as iron and manganese oxides settle and react with HS^-.

Jørgensen and co-workers (*1*, *21–23*) have demonstrated that the $S_2O_3^{2-}$ shunt plays an important role in coupling the oxidative and reductive pathways of the sulfur cycle in both marine and freshwater environments. The discovery of an abundant group of organisms that can utilize $S_2O_3^{2-}$ and other partially oxidized sulfur species as electron acceptors provides additional biological mechanisms for establishing electron flow through sulfur compounds. These findings indicate that there is a direct, enzymatic connection between marine sulfur and carbon cycles, which is separate from SO_4^{2-} reduction.

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Multiple Evolutionary Origins of Magnetotaxis in Bacteria

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Magnetosomes are intracellular, iron-rich, membrane-enclosed magnetic particles that allow magnetotactic bacteria to orient in the earth's geomagnetic field as they swim. The magnetosomes of most magnetotactic bacteria contain iron oxide particles, but some magnetotactic species contain iron sulfide particles instead. Phylogenetic analyses of small subunit ribosomal RNA sequences showed that all known magnetotactic bacteria of the iron oxide type are associated with the α subgroup of the Proteobacteria in the domain Bacteria. In contrast, uncultured magnetotactic bacteria of the iron sulfide type are specifically related to the dissimilatory sulfate-reducing bacteria within the δ subdivision of the Proteobacteria. These findings indicate a polyphyletic origin for magnetotactic bacteria and suggest that magnetotaxis based on iron oxides and iron sulfides evolved independently.

Magnetotaxis in bacteria (*1*) is the result of a permanent magnetic dipole moment that causes a cell to be oriented in the earth's geomagnetic field as it swims (*2*). The magnetic dipole moment is caused by the presence of magnetosomes (*3*), intracellular, iron-rich, membrane-enclosed magnetic particles. In most magnetotactic bacteria, the particles consist of the ferrimagnetic iron oxide magnetite (Fe_3O_4) (*4–8*). However, some magnetotactic bacteria collected from sulfidic, brackish-to-marine aquatic habitats have recently been found to contain iron sulfide particles (*9*, *10*), including ferrimagnetic greigite (Fe_3S_4) (*9*, *11*, *12*) and nonmagnetic pyrite (FeS_2) (*9*, *11*). Although magnetotactic bacteria of both the iron oxide and iron sulfide types are frequently found in the same habitats, they grow in discrete locations, as indicated

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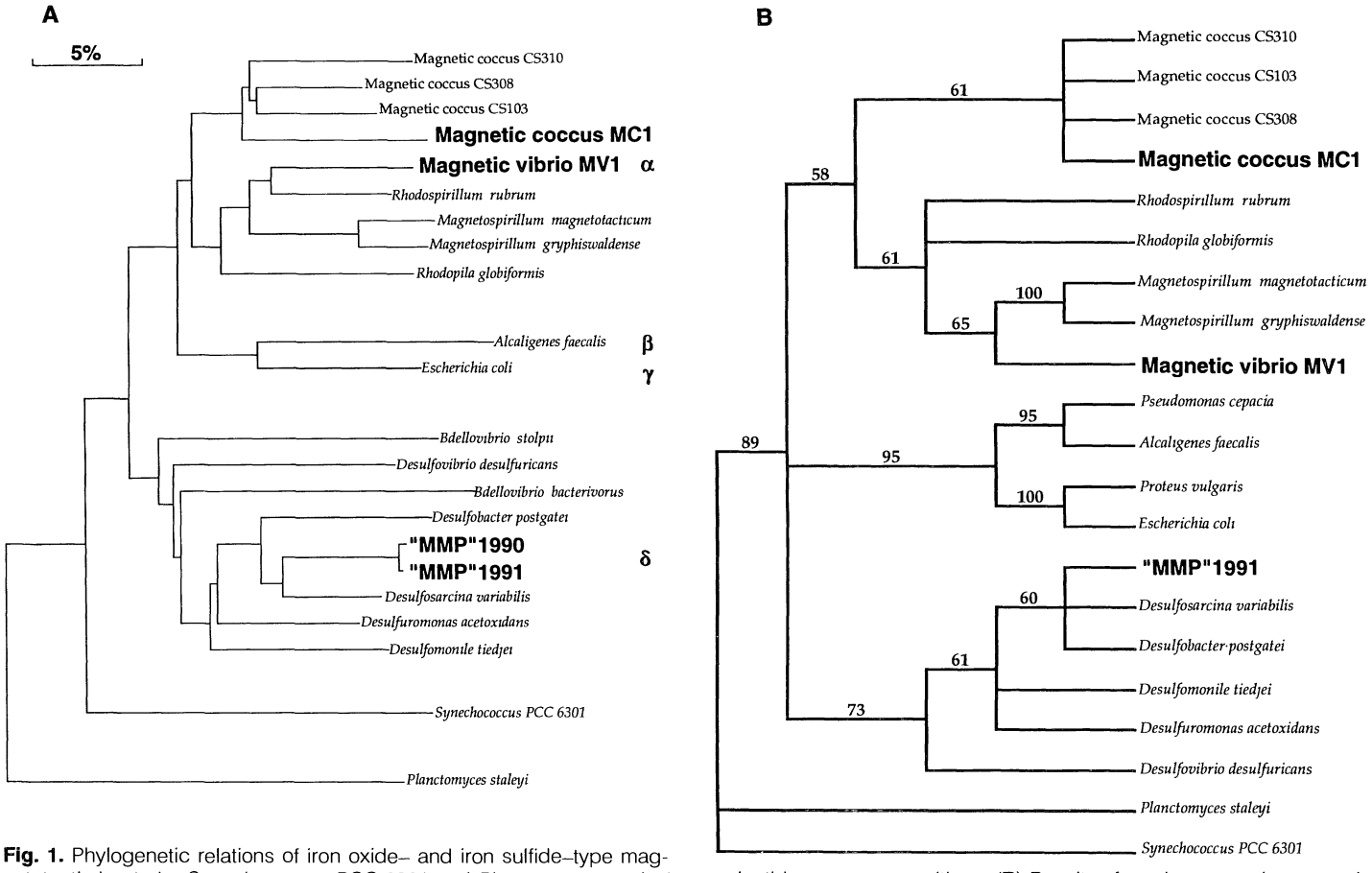

Fig. 1. Phylogenetic relations of iron oxide– and iron sulfide–type magnetotactic bacteria. *Synechococcus* PCC 6301 and *Planctomyces staleyi* are included as outgroups to the Proteobacterial lineage. (**A**) Evolutionary distances were estimated with the correction of Jukes and Cantor (*27*) from 630 aligned, moderately to highly conserved nucleotide positions. Phylogenetic tree topology was inferred with the least squares distance-matrix analyses software of Olsen (*28*) obtained from the ribosomal RNA database project (*29*). Scale bar represents five fixed mutations per 100-nucleotide sequence positions. (**B**) Results of maximum-parsimony analysis (PAUP version 3.0s) and bootstrap resampling of the dataset (*30–32*). The tree topology summarizes the results obtained from 100 trees generated from 100 individual bootstrap resamplings of the dataset. Values indicate the percentage of bootstrap resamplings that yielded the branching pattern to the right of that value in the majority-rule consensus tree. Sequences (*33*) obtained in this study are in bold type.

by vertical oxygen and sulfide concentration gradients (*13*). However, iron sulfide–type magnetotactic bacteria have not been cultivated to date, and their relation to iron oxide–type magnetotactic bacteria has not been described.

Magnetotactic bacteria were collected from sulfidic, brackish-to-marine sediment and water from various coastal sites in New England with the use of a modified racetrack technique (*14*). A magnetotactic, many-celled prokaryote (MMP) (*15, 16*) was collected in large numbers, but could not be cultured. Transmission electron microscopy and energy-dispersive x-ray analysis of the magnetosomes in this organism showed that the magnetosomes contained only iron and sulfur, consistent with the presence of greigite and pyrite (9). Three magnetotactic cell types were also isolated and cultured for comparative purposes. These included the first coccoid strain to be cultured axenically, designated MC-1 (*17*), and two vibrioid strains, designated MV-1 (8) and MV-2 (*17*). Transmission electron microscopy and electron diffraction have determined that strains MC-1 and MV-2 contain particles of magnetite (*18*).

Nucleic acids were purified from magnetotactic bacteria, and the small subunit ribosomal RNA genes (ssu rRNA) were amplified, cloned, and sequenced (*19*). Distance-matrix (Fig. 1A) and maximum-parsimony analyses (Fig. 1B) of the ssu rRNA sequences of the cultured, iron oxide–type magnetotactic bacteria indicated that these bacteria were members of the α subdivision of the Proteobacteria (*20*) in the domain Bacteria. The rRNA sequences of the magnetite-containing vibrioid strains MV-1 and MV-2 were identical and fell within the α subdivision. However, these strains were not closely related to any previously characterized magnetotactic species, including the magnetotactic spirilla *Magnetospirillum* (formerly *Aquaspirillum*) *magnetotacticum* (*21*) and *M. gryphiswaldense* (*22*) or to several uncultured magnetotactic cocci (*23*). Unrestricted rRNA sequence similarity values between strain MV-1 and other α Proteobacteria ranged from 0.83 to 0.87. The magnetite-containing coccus, strain MC-1, was more peripherally related to the α Proteobacteria group. Ribosomal RNA sequence similarities between strain MC-1 and its closest relatives, a group of different, uncultured, freshwater magnetotactic cocci (*23*) (CS103, CS308, and CS310; Fig. 1), ranged from 0.85 to 0.87. Secondary structural analyses support the inclusion of the magnetite-containing magnetotactic bacteria within the α subdivision of the Proteobacteria. For example, strain MC-1 contained a 3–base pair stem between nucleotide positions 184 to 193 (*Escherichia coli* numbering) and a shortened helix between nucleotide positions 198 to 219. These combined features distinguish members of the α subdivision from those of the β, γ, and δ subdivisions of the Proteobacteria (*24*).

Phylogenetic analyses of the ssu rRNA sequence of the MMP indicated the affiliation of this bacterium with the δ subdivision of the Proteobacteria (Fig. 1). This

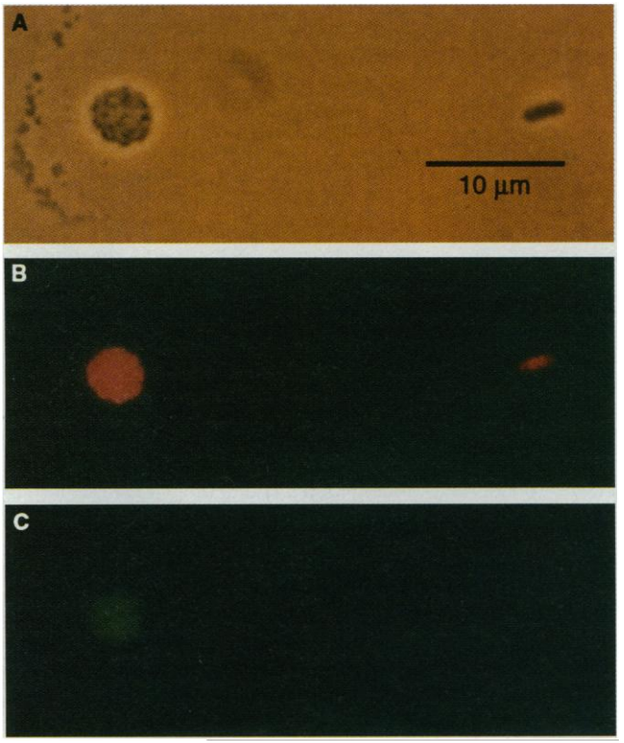

Fig. 2. (**A**) Phase contrast and (**B**) epifluorescent micrographs of the same viewing field of a magnetic enrichment containing the MMP. Cells were fixed in formalin and hybridized (*34*) simultaneously with a Texas red–labeled (Molecular Probes, Eugene, Oregon), eubacterial-specific oligonucleotide probe (*25*) and a fluorescein-labeled, MMP-specific probe. (B) Fluorescence micrograph with epifluorescence filters specific for Texas red excitation and emission spectra. (**C**) Fluorescence micrograph with epifluorescence filters specific for fluorescein excitation and emission spectra. The sequence of the MMP-specific probe was GGA CTC ACC CTT AAA CGG. This sequence is complementary to nucleotide positions 218 to 235 (*E. coli* numbering) of the MMP rRNA.

iron sulfide–type magnetotactic bacterium was specifically related to the dissimilatory sulfate-reducing Proteobacteria and shared the highest rRNA sequence similarity with *Desulfosarcina variabilis* (unrestricted rRNA sequence similarity = 0.91; Fig. 1). Direct sequencing of the polymerase chain reaction (PCR)–amplified ribosomal DNA obtained from a magnetically enriched, monotypic cell collection containing only the morphologically conspicuous MMP showed the amplification of only one rRNA sequence type. The sequences of ssu rRNA gene clones obtained from two separate magnetically enriched samples [obtained September 1990 (MMP 1990; Fig. 1) and August 1991 (MMP 1991; Fig. 1)] showed greater than 99% similarity to one another and to the direct PCR sequence. A fluorescein-labeled oligonucleotide probe complementary to an 18-nucleotide diagnostic region within this rRNA sequence was used to verify the phylogenetic identity of the MMP. All magnetotactic morphotypes in magnetically enriched samples bound a Texas red–labeled, eubacterial-specific probe (*25*) (Fig. 2B), indicating that these cell types contained intact and accessible target rRNA. However, only the MMP cells bound the MMP-specific oligonucleotide probe labeled with fluorescein (Fig. 2C).

The type of magnetosome mineral appears to reflect phylogenetic affiliation. All iron oxide–type magnetotactic bacteria examined in this study and elsewhere (*21–23*) are specifically affiliated with the α subdivision of the Proteobacteria. In contrast, the iron sulfide–type MMP is affiliated with the dissimilatory sulfate-reducing bacteria in the δ subdivision of the Proteobacteria. The data suggest that iron sulfide–type magnetotactic bacteria (*11, 12*) other than the MMP are also probably associated with the dissimilatory sulfate-reducing Proteobacteria. Because the subdivisions of the Proteobacteria are comprised of four distinct, coherent phylogenetic lineages that have diverged at an unknown time in the past (*24, 26*), it appears that magnetotaxis based on iron oxides and iron sulfides has separate evolutionary origins. Moreover, the biochemical basis for biomineralization and magnetosome formation for iron oxide–type and iron sulfide–type bacteria are likely fundamentally different. The advantage conferred by magnetotaxis is presumably an increased efficiency in finding and maintaining position relative to chemical and redox gradients. It is remarkable that both types of magnetotactic bacteria converged on similar solutions to the problem of magnetotaxis—construction of a permanent magnetic dipole moment sufficient to orient the cell in the geomagnetic field by using different magnetic minerals.

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Probing the Structure and Mechanism of Ras Protein with an Expanded Genetic Code

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Mutations in Ras protein at positions Gly^{12} and Gly^{13} (phosphate-binding loop L1) and at positions Ala^{59}, Gly^{60}, and Gln^{61} (loop L4) are commonly associated with oncogenic activation. The structural and catalytic roles of these residues were probed with a series of unnatural amino acids that have unusual main chain conformations, hydrogen bonding abilities, and steric features. The properties of wild-type and transforming Ras proteins previously thought to be uniquely associated with the structure of a single amino acid at these positions were retained by mutants that contained a variety of unnatural amino acids. This expanded set of functional mutants provides new insight into the role of loop L4 residues in switch function and suggests that loop L1 may participate in the activation of Ras protein by effector molecules.

The proteins encoded by the mammalian *ras* proto-oncogene act as a molecular switch, passing extracellular signals for cell growth and differentiation to one or more intracellular effector molecules (*1–4*). The chemical basis for switch function involves the cycling of the protein between the inactive guanosine diphosphate (GDP)–bound state and the active guanosine triphosphate (GTP)–bound state. Point mutations that result in a decrease in the intrinsic guanosine triphosphatase (GTPase) activity of Ras or the GTPase activity stimulated by GTPase-activating protein (GAP) (*5–8*) are associated with approximately 30% of human tumors. To gain greater insight into the molecular mechanisms of switch inactivation (and oncogenic activation), we systematically substituted residues in loop L1 (the phosphate-binding loop) and loop L4 (the switch II region) with a series of unnatural amino acids (Figs. 1 and 2). The ability to precisely vary the steric or electronic properties of a given residue (*9–14*) has allowed us to test mechanistic and structural issues not addressable by conventional mutagenesis studies.

Incorporation of unnatural amino acids into Ras protein was accomplished by in vitro suppression of TAG nonsense mutations with a chemically aminoacylated suppressor tRNA (*15–17*). As a control, wild-type protein and a $Gly^{12} \rightarrow Pro$ mutant were generated in vivo and in vitro by suppression of the $Gly^{12} \rightarrow TAG$ mutant with Gly and Pro, respectively (Fig. 3). The resulting proteins were purified to homogeneity from in vitro transcription-translation reactions (1 ml) in approximately 20% yield by sequential chromatography on DEAE-sephacel and an immunoaffinity column. The purified proteins had the same chromatographic properties, intrinsic GTPase activity, and GAP-stimulated GTPase activity as the corresponding Ras protein synthesized in vivo (Table 1). In vitro protein synthesis reactions with wild-type plasmid (pRG) yielded active protein (40 to 50 μg/ml); suppression efficiencies are listed in Table 1. In vitro synthesis reactions that contained the nonsense mutants in the absence of suppressor tRNA or in the presence of full-length unacylated suppressor $tRNA_{CUA}$ yielded less than 0.5% of Ras protein. The amount of Ras synthesized was determined by polyacrylamide gel electrophoresis, immunoprecipitation of [^{35}S]methionine-labeled protein, and GTPase activity assays (Fig. 3).

Mutations at Gln^{61} are among the most common that lead to impaired intrinsic GTPase activity and, in many cases, oncogenic activation (*18*). On the basis of biochemical studies (*19*) and the three-dimensional x-ray crystal structures of the GTP-, Gpp(CH_2)p-, and Gpp(NH)p-bound forms of Ras (*20–27*), it has been proposed that Gln^{61} is critical for γ-phosphate binding and catalysis. Specifically, it has been proposed that the γ-carboxamide of Gln^{61} either polarizes water 175 for attack on the γ-phosphate (*24*) or stabilizes the incipient pentacoordinate transition state (*25*). In order to test these mechanistic hypotheses, we substituted Gln^{61} with the Gln homolog homoglutamine (HGln) or with the isoelectronic, isosteric nitro analog (NGln) (Table 1 and Fig. 2). Whereas 17 natural mutants at position 61 have reduced GTPase activity and are not activated by GAP, the $NGln^{61}$ mutant had GTPase activity similar to that of the wild-type protein and was activated by GAP. The fact that this mutant retained activity even though the nitro group is a much poorer base than a carboxamide group [the protonated form of the nitro group has a pK_a (negative loga-

Fig. 1. Model of Ras protein with bound GTP (*25*).

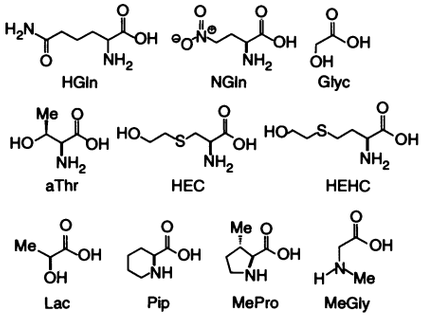

Fig. 2. Structures of amino acids (*47*). The α-amino group of all the amino acids was protected as the NVOC derivative, whereas the α-hydroxyl group of the hydroxy acids was protected as the *o*-nitrobenzyl derivative. The free acids were activated for acylation to pdCpA as cyanomethyl esters (*15*). Me, methyl group.

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