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Evidence for two types of subunits in the bacterioferritin of *Magnetospirillum magnetotacticum*

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Abstract

In order to investigate the role of bacterioferritin (Bfr) in the biomineralization of magnetite by microorganisms, we have cloned and sequenced the *bfr* genes from *M. magnetotacticum*. The organism has two *bfr* genes that overlap by one nucleotide. Both encode putative protein products of 18 kDa, the expected size for Bfr subunits, and show a strong similarity to other Bfr subunit proteins. By scanning the DNA sequence databases, we found that a limited number of other organisms, including *N. gonorrhea, P. aeruginosa*, and *Synechocystis* PCC6803, also have two *bfr* genes. When the sequences of a number of microbial Bfrs are compared with each other, they fall into two distinct types with the organisms mentioned above having one of each type. Differences in heme- and metal-binding sites and ferroxidase activities of the two types of subunits are discussed. © 1997 Elsevier Science B.V.

Keywords: Ferroxidase; Heme; Homology; Magnetite; Sequence

1. Introduction

Magnetotactic bacteria were first observed by Blakemore (1975). The organism, *Magnetospirillum magnetotacticum* (Mm), is a microaerophilic, Gram-negative, motile spirillum, belonging to the α subdivision of the proteobacteria. Its most remarkable property is its ability to convert iron into single domain crystals of the mineral magnetite (Fe_3O_4) (Blakemore, 1982). The crystals are arranged in long chains, called 'magnetosomes', and are oriented so that the magnetic moments are additive, and the magnetosome functions as a bar magnet. The entire magnetosomal structure is enclosed in a protein-containing lipid bilayer, the magnetosomal membrane (Gorby et al., 1988). Since then, a number of other magnetotactic microorganisms have been isolated and/or described (Bazylinski et al., 1988; Farina et al., 1990), but the mechanism by which these organisms convert iron to magnetite has not been elucidated as yet.

Both eukaryotes and prokaryotes store large amounts of iron in specialized structures composed of the proteins ferritin (Fr) or bacterioferritin (Bfr), respectively. Because of similarities in the proteins, they are considered to be members of a larger superfamily (Andrews et al., 1991; Grossman et al., 1992) that includes 'Fr-like' proteins found in prokaryotes (Izuhara et al., 1993), and some DNA-binding proteins (Peña and Bullerjahn, 1995). Typically, the Fr structure consists of 24 subunits of 17–19 kDa, assembled into a hollow shell. In the case of mammalian Fr, there are two types of subunits, designated the H- and L- chains. In the case of *E. coli* (Ec), the Bfr consists of 24 identical subunits. Up to 4000 iron atoms may be incorporated into the cavity to form an internal ferrihydrite (Fh; $5Fe_2O_3.9H_20$) core.

Indirect evidence suggests that Fr also may be involved in the biomineralization of magnetite. The

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Abbreviations: aa, amino acid(s); Asp, aspartic acid; Av, *Azotobacter vinelandii*; Bfr, bacterioferritin; *bfr1* and *bfr2*, genes coding for bacterioferritin subunits; bp, base pair(s); Bm, *Brucella melitensis*; Ec, *Escherichia coli*; Fh, ferrihydrite; Fr, ferritin; Glu, glutamic acid; His, histidine; kb, kilobase(s) or 1000 bp; Ma, *Mycobacterium avium*; Ml, *Mycobacterium leprae*; Mm, *Magnetospirillum magnetotacticum*; Met, methionine; Ng, *Neisseria gonorrhea*; nm, nanometer; nt, nucleotide(s); ORF, open reading frame; Pa, *Pseudomonas aeruginosa*; Pp, *Pseudomonas putida*; RBS, ribosome binding site; Rc, *Rhodobacter capsulatus*; Sy, *Synechocystis* sp.; Tyr, tyrosine.

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Polyplacophoran mollusks (the chitons) accumulate magnetite in the radular teeth, presumably to harden them for grazing off of rocks. The magnetite is always formed by conversion of pre-existing Fh (Kirschvink and Lowenstam, 1979; Lowenstam and Weiner, 1989), and Fr is involved in this process (Nesson and Lowenstam, 1985). Studies of Mm have revealed the presence of a low-density ferric oxide, a high-density hydrous ferric oxide (probably Fh) and magnetite within the intact cells (Frankel and Blakemore, 1984). Hence, these bacteria may use a biochemical pathway similar to that of the Polyplacophoran mollusks in magnetite formation (Kirschvink and Lowenstam, 1979). However, an analytical high-resolution transmission electron microscopic (HRTEM) study by Vali and Kirschvink (1990) on magnetotactic bacteria failed to detect the presence of microcrystalline iron particles larger than a few nanometers in size anywhere except within the magnetosomes. They concluded that no Fr cores were present within the cytoplasm of the cells and speculated that the magnetotactic bacteria might have lost the ability to produce Bfr or that it could not be distinguished from the magnetosomes. Hence, a clear target for determining whether or not magnetite biomineralization operates through similar pathways in mollusks and bacteria is to determine whether the magnetotactic bacteria retain the *bfr* genes.

Using PCR and DNA hybridization, we have now identified two overlapping *bfr* genes in Mm. One gene, *bfr1*, encodes a Bfr subunit with strong similarity to the single Bfr subunit found in Ec (Andrews et al., 1989), *A. vinelandii* (Grossman et al., 1992), and many other organisms, whereas the product of the second gene, *bfr2*, has more identity with one of the two Bfr subunits found in a few organisms such as *P. aeruginosa* (Pa; Moore et al., 1994), *Synechocystis* PCC6803 (Sy; Kaneko et al., 1996), and *N. gonorrhea* (Ng; Chen, C.-Y., 1996, GenBank Accession Nos U76633 and U76634). A comparison of the structures of these two groups of proteins indicates that they constitute two distinct families. Some structural features of the two types of subunits are compared and discussed.

2. Experimental and discussion

2.1. Nucleotide sequences of bfr1 and bfr2

A 2306-bp *Eco*RI DNA fragment, cloned from Mm, was sequenced (Fig. 1) and found to contain two ORFs, the first starting at bp 766 and continuing to bp 1257, and the second starting at bp 1257 and continuing to bp 1736. Thus, the ORFs overlap by one nucleotide.

Little is known so far about the regulation of the genes. There are no very obvious transcriptional start signals in regions upstream of either of the ORFs. There are putative RBSs at bp 755–758 for *bfr2* and at 1247–1250 for *bfr1* and several translational in-frame stop codons found at the end of each gene.

Genes that are involved in the uptake of iron may be repressed by a high iron concentration due to the activity of the repressor, FUR (Bagg and Neilands, 1987), which is activated by excess iron. Thus, the presence of FUR-binding sites overlapping the promoter regions is an indication that a gene is regulated by iron concentration. Two putative FUR-binding sites with better than 50% identity with the FUR-box consensus sequence can be found in the *bfr2* sequence, although they are within the gene at bp 1065–1083 and bp 1098–1116. A similar arrangement is found in the Av *bfr* gene (Grossman et al., 1992).

2.2. Two types of Bfr subunits

Gene *bfr1* encodes a putative protein of 160 aa residues and *bfr2*, a protein of 164 aa residues, giving molecular weights of about 18 kDa, typical for Bfr subunits. When a database search was made using the aa sequences of the ORFs, they both showed a strong identity to other Bfr sequences (Fig. 2). However, the Bfr1 subunit showed more identity with Ec Bfr (62%) than it did with Bfr2 (43%). Both subunits have a weak similarity to the 'ferritin-like ' proteins described for Ec and to the eukaryotic ferritin H- or L-chains (comparisons not shown).

When a wider search was made for other organisms having Bfr2-like subunits, four were found. Sy, Ng, and Pa each have two types of subunits, and in each case, one has a greater identity with Bfr1 and the other with Bfr2 (Fig. 2). In the case of the fourth organism, Pp, only a *bfr2*-like gene has been entered in the database so far. In none of the cases where an organism has two *bfr* genes have they been reported to be overlapping.

When these sequences were entered into the program PILEUP, which compares them pairwise to establish sequence similarities, the plot produced a tree with two main branches (Fig. 3). Thus, the Bfr sequences could be divided into two types with all of the members of one type showing a greater identity with each other than with the other type.

We have no information so far on whether both types of subunits are produced in Mm, but we expect that antibodies specific for the two subunits will tell us if both are produced and in what proportion. In the case of Pa, which also has two genes, published studies suggest that the Bfr proteins are indeed composed of heterologous subunits in varying proportions (Moore et al., 1994). Thus, although Ec has a Bfr protein made up of 24 identical subunits, there are other organisms that produce more than one subunit and which most

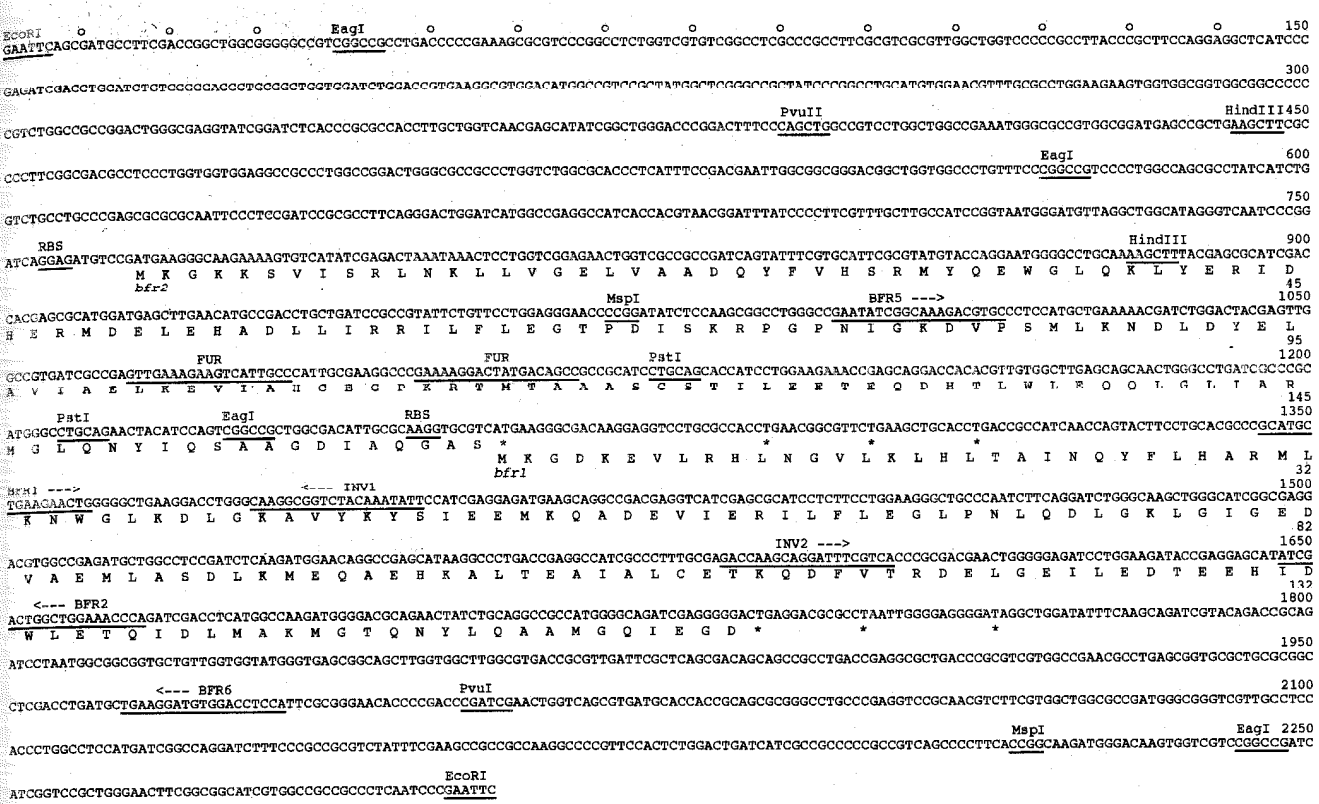

Fig. 1. The 2.3-kb *Eco*RI insert containing genes *bfr1* and *bfr2*. Total genomic DNA, prepared from Mm (strain MS-1; ATCC 31632), was subjected to both direct and inverse PCR, using oligonucleotides synthesized by the Biopolymer Synthesis and Analysis Resource Center (Caltech) and a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA), following the program described in Sambrook et al. (1989). The first oligonucleotides used, BFR1 (5′-CGCATGTATIAAIACTGGGG-3′) and BFR2 (5′-TGCGTTTCCAGCCAITCGAT-3′) produced a sequence of 323 bp. This was followed by inverse PCR, using oligonucleotides INV1 and INV2, on genomic DNA digested with the restriction enzyme MspI and then ligated under dilute conditions, to obtain the sequence between the MspI sites. A 988-bp sequence that included the *bfr1* gene was generated by the oligonucleotides BFR5 and BFR6, cloned into the pCRII vector using the Invitrogen (San Diego, CA) TA Cloning Kit and used as a probe for a partial small insert library, which was obtained by digesting Mm DNA with the restriction enzyme, *Eco*RI (NE Biolabs, Beverly, MA), ligating the fragments into the Bluescript II KS ± vector (Stratagene, La Jolla, CA), transforming the resultant plasmids into XL-1 bacteria (Stratagene), and preparing dotblots, as described in Sambrook et al. (1989) from single white colonies. The dotblots were probed with the α^{32}P-dCTP-labelled 988-bp sequence and DNA prepared from positive clones using a Spin Plasmid Kit (Qiagen, Chatsworth, CA). DNA sequencing was carried out by the DNA Sequencing Core Facility at Caltech. The complete sequence of the DNA fragment was determined by using suitable primers (Biopolymer Synthesis and Analysis Resource Center) placed every 300 bp along the template in both directions. The sequence has been deposited with GenBank under the Accession No. AF001959. Some of the restriction enzyme sites and the putative RBS sites and FUR boxes are underlined and identified. The two ORFs with sequence similarity to other Bfr proteins are translated into aa. Stop codons are indicated by asterisks.

likely have Bfr proteins containing mixtures of the subunits. In higher organisms, of course, Fr is composed of two types of subunits, the H- and L-chains. The existence of two types of subunit could lead to divergent function and might therefore result in a Bfr protein with properties different from that formed by a single-subunit Bfr. Two such differences can be noted.

2.3. Heme binding

One of the major differences between ferritins is that Bfr contains heme, whereas Fr does not. Initially, it was thought that the number of heme groups was limited to 12 per 24 subunits (Stiefel and Watt, 1979). More recently, however, Bfrs containing up to 24 heme groups have been described (Kadir and Moore, 1990), although the presence or absence of heme apparently has no effect on the iron storage capability of the protein (Andrews et al., 1995). Whether it has an effect on the structure of the iron deposited is unknown.

Methionine has been implicated as the ligand that holds the heme group in place, and Met52, which is highly conserved in the Bfr1-like subunits (see Fig. 2), is thought to play this role (Frolow et al., 1994). In the structure of Ec Bfr, the subunits form pairs related by twofold symmetry with a single heme group embedded between two directly opposing Met52 residues (Frolow et al., 1994). In the Bfr2 type subunit, however, the residue at position 52 is not Met, although in each case, there is a Met residue closeby at position 48 or 49 (Fig. 2). Thus, if the Bfr2-type subunits also form pairs with twofold symmetry, the potential heme-binding Met

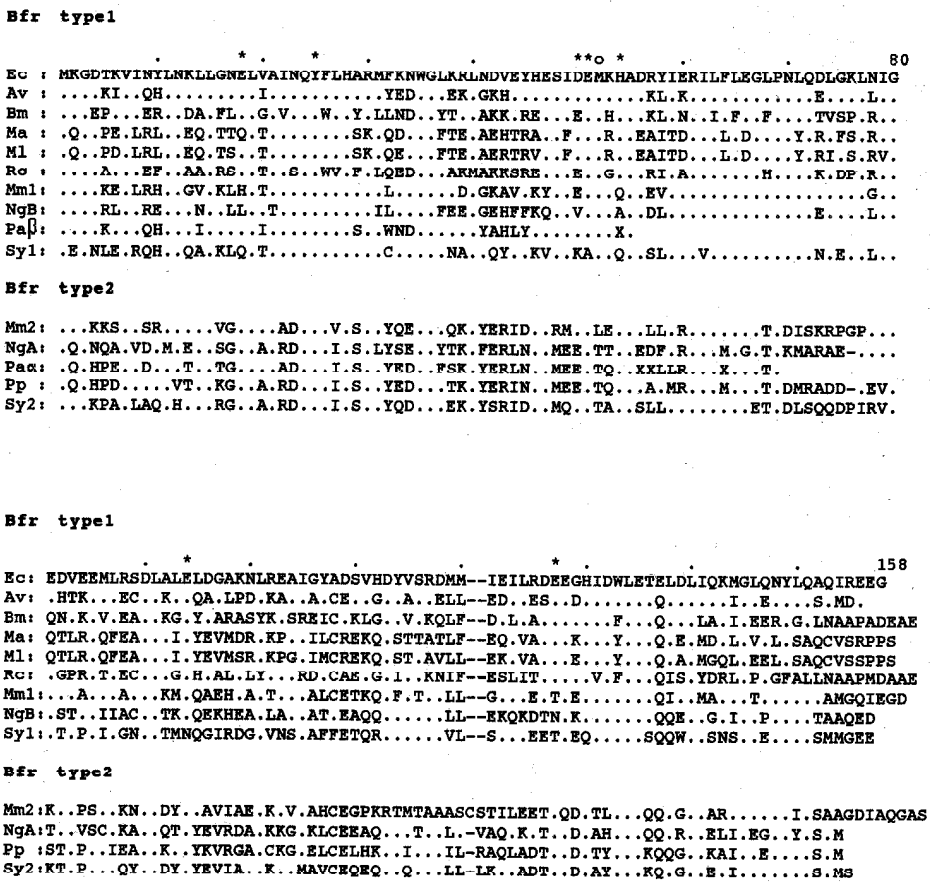

Fig. 2. The aa sequences of Bfr subunit types 1 and 2 compared to the Bfr sequences in some other organisms: Ec (Andrews et al., 1989), Av (Grossman et al., 1992), Bm (Denoel et al., 1995), Ma (Inglis et al., 1994), Ml (Pessolani et al., 1994), Rc (Penfold et al., 1996; GenBank Accession No. U66717), Ng (Chen, 1996, GenBank Accession Nos. U76633 and U76634), Pa (Moore et al., 1994), Pp (Anderson et al., 1996; GenBank Accession No. Z54247) and Sy (Kaneko et al., 1996). Sequences were obtained using the FETCH program of the Genetics Computer Group (GCG) and homology searches made using the BLAST network service of the National Center for Biotechnology Information. The two types of subunit in Mm are called 1 and 2; in Ng, A and B and in Pa, α and β. We have arbitrarily designated the Sy subunits as 1 and 2. Only partial sequences of the Pa subunits are available so far. One or two gaps were introduced to improve the alignment of some sequences. The numbering used refers to that of Ec and only residues not identical with the Ec sequence are noted. Met52 is indicated by an 'o' and the ferroxidase sites by an '*'.

residues at positions 48 or 49 would not be directly opposite each other and each might then be able to bind a heme group. This might explain the increased heme-binding capacity observed by Kadir and Moore (1990) in Pa.

2.4. Ferroxidase activity

Fr has a ferroxidase activity that mediates the growth of the iron core by oxidizing Fe^{2+} to Fe^{3+}. The aa residues involved in this activity, as well as the specific metal-binding sites, form a ferroxidase center. Ferr oxi dase activity, which is present in the mammalian H-chain, is lacking from the L-chain, which has lost many of the ferroxidase sites (Levi et al., 1988). In most Bfrs, these same aa residues—Glu18, Tyr25, Glu(Asp)50, Glu51, His54, Glu94, and Glu127—that confer ferroxidase activity are well conserved (Andrews et al., 1991). In organisms that have two *bfr* genes, however, one might expect to find some evolution toward different functions of the subunits. In the organisms listed in Fig. 2 that have a single type of Bfr subunit, the residues associated with ferroxidase activity are well conserved. In the case of organisms with two genes, the residues in the type 2 subunit are also very well conserved, but the type 1 subunits in these strains show deviations (Fig. 2). Mm, Ng, and Sy, each have lost two to four of the conserved aa. Thus, the type 1 subunit in these strains appears to be losing its ferroxidase activity and becoming more L-chain-like.

Very preliminary molecular modelling (Peitsch, 1996) indicates that the region from aa 113 to 125 in Mm Bfr2 is completely different from that of other Bfr subunits examined so far (Fig. 2). As this region lies between Glu94 and Glu127, which are part of the binuclear metal-binding site in the ferroxidase center

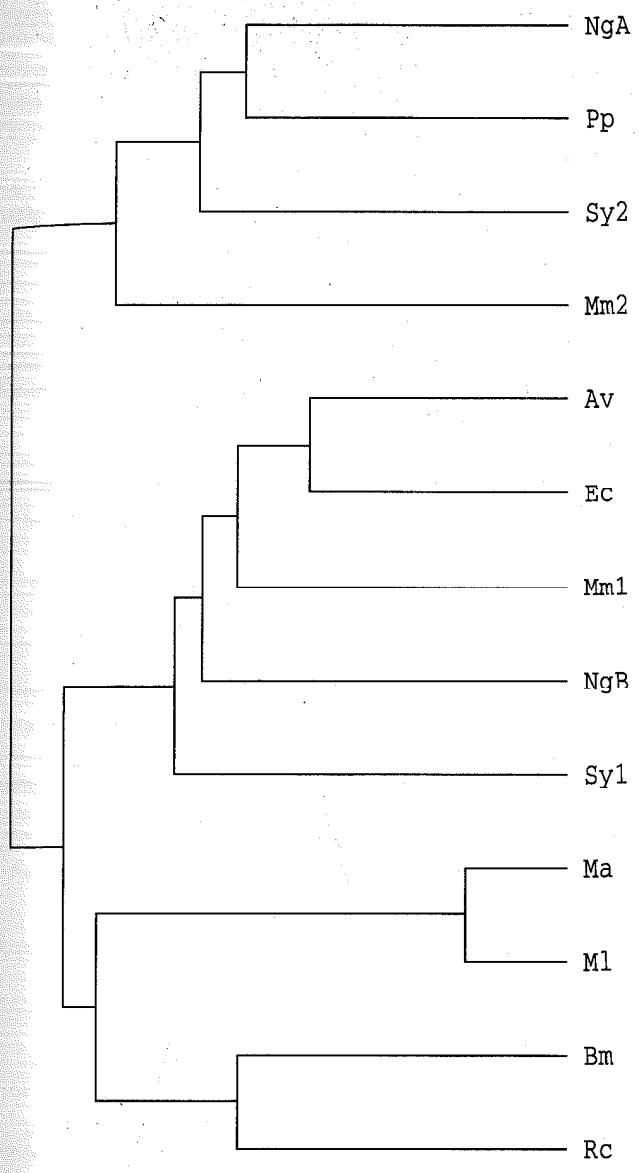

Fig. 3. Similarities of the Bfr aa sequences among various strains. The sequences of the strains listed in Fig. 2 were compared using the PILEUP program of the GCG. The partial sequences of Pa were not included.

(Frolow et al., 1994), its relevance for the biomineralization of magnetite deserves further study.

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