Anion-independent Iron Coordination by the Campylobacter jejuni Ferric Binding Protein*

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Campylobacter jejuni, the leading cause of human gastroenteritis, expresses a ferric binding protein (cFbpA) that in many pathogenic bacteria functions to acquire iron as part of their virulence repertoire. Recombinant cFbpA is isolated with ferric iron bound from Escherichia coli. The crystal structure of cFbpA reveals unprecedented iron coordination by only five protein ligands. The histidine and one tyrosine are derived from the N-terminal domain, whereas the three remaining tyrosine ligands are from the C-terminal domain. Surprisingly, a synergistic anion present in all other characterized ferric transport proteins is not observed in the cFbpA iron-binding site, suggesting a novel role for this protein in iron uptake. Furthermore, cFbpA is shown to bind iron with high affinity similar to Neisserial FbpA and exhibits an unusual preference for ferrous iron (oxidized subsequently to the ferric form) or ferric iron chelated by oxalate. Sequence and structure analyses reveal that cFbpA is a member of a new class of ferric binding proteins that includes homologs from invasive and intracellular bacteria as well as cyanobacteria. Overall, six classes are defined based on clustering within the tree and by their putative iron coordination. The absence of a synergistic anion in the iron coordination sphere of cFbpA also suggests an alternative model of evolution for FbpA homologs involving an early iron-binding ancestor instead of a requirement for a pre-existing iron-binding ancestor.

The obstacles that prokaryotes face in the acquisition of growth essential iron are substantial. Within anoxic environment, available iron is limited because of its low solubility. Iron is further sequestered in animals by host iron-binding proteins, such as transferrin and lactoferrin (1, 2). Survival therefore

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| Table I: Data collection and refinement statistics for cFbpA |
|-----------------|-----------------|
| Crystal         | cFbpA           |
| Resolution range (Å) | 16.0–1.8 (1.86–1.80) |
| Rmerge (I/|I|) | 0.054 (0.254) |
| Completeness (%) | 93.8 (91.4) |
| Total reflections | 365222 |
| Unique reflections | 17929 |
| Rwork | 0.164 |
| Rfree | 0.203 |
| Root mean square deviation bonds | 0.013 |
| Overall B factor (Å²) | 15.9 |

*Numbers in parentheses refer to the highest resolution shell.

Materials and Methods

Cloning and Expression of cFbpA from C. jejuni NCTC 11168—Purified genomic DNA was obtained from Dr. Erin Gaynor (University of British Columbia). The coding region of cFbpA, lacking the signal sequence, was amplified by PCR using the 5’ primer (5’-AAGCTTCCATGGCTGACCTGAACTGGG-3’) and the 3’ primer (5’-ATATCGGAGCCGCTTCTACTATCCAGGCTTGCAATTT-3’). The primers include NcoI and XhoI restriction sites, for cloning into a pET28a expression vector (Novagen, Madison, WI) in-frame with a C-terminal His tag. The 3’ primer also includes a Factor Xa site for removal of the His tag.

Recombinant expression was performed in Escherichia coli HMS174(DE3) cells (Novagen). Growth medium consisted of 2× YT (Difco) supplemented with 25 μg/ml kanamycin. Inoculated cultures were grown initially at 37 °C and then transferred to 30 °C. The cells were induced at an A600 of 0.5–0.6 with 0.5 mM isopropyl β-D-thiogalactopyranoside and grown overnight. The cells were lysed at 4 °C using an Avestin EmulsiFlex-C5 homogenizer. The soluble fraction was applied to a nickel column and eluted using imidazole. The histidine tag was removed by digestion with Factor Xa (Hematologics) in 150 mM NaCl, 2 mM CaCl2, 60 mM sodium acetate. Each drop was made from an equal volume of excess EDTA in 10 mM Tris-HCl, pH 7.4, for 10 min. The buffer was exchanged with 200 mM NaCl and 10 mM Tris-HCl by gel filtration.

Crystal Structures—Holo cFbpA crystals were grown at 19 °C by the hanging drop vapor diffusion method and microseeding. The reservoir contained 24% polyethylene glycol 4000, 0.1 M Tris-HCl, pH 8, and 60 mM sodium acetate. Each drop was made from an equal volume of reservoir and a 15 mg/ml protein stock solution. The crystals were transferred to the reservoir plus 30% (v/v) glycerol and looped directly into a cryostream at 100 K. X-ray data were collected on a home labo-

RESULTS

cFbpA Structure—The overall structure of cFbpA consists of two globular domains linked by two β-strands (Fig. 1A). The N-terminal domain is comprised of residues 5–103 and 239–283. The remaining residues, 104–238 and 284–321, form the C-terminal domain. Each domain is formed from α-β-α units that contribute to form a central five-stranded β-sheet surrounded by six α-helices in the N-terminal domain and a central three-stranded β-sheet surrounded by eight α-helices in the C-terminal domain. The hinge region, which may allow for domain movement, is located within the two β-strands. Ferric binding proteins are typically monomers, and the two molecules in the asymmetric unit are essentially the same (0.26 Å root mean square deviation on α-carbons) and make few contacts. All of the subsequent results are for molecule A.

The iron-binding site is located at the domain interface, and the five coordinating residues originate from both domains (Fig. 1B). The coordination sphere is composed of His14 near the N terminus and four Tyr residues: one from the N-terminal domain (Tyr15) and three from the C-terminal domain (Tyr146, Tyr202, and Tyr203). His14 and Tyr15 are part of a long loop, whereas Tyr146, Tyr202, and Tyr203 are contributed from the N termini of two α-helices. The iron is coordinated in a distorted octahedral geometry with one absent site (see Table S1).

Iron Binding Studies—The visible spectroscopic data were obtained on a Varian Cary Bio50 UV spectrophotometer using a 1-cm-path length 80-μl quartz cuvette at room temperature. All of the reagents used were purchased from Sigma-Aldrich. Holo nFbpA was obtained as described previously (26). All of the iron binding experiments were performed using 2 mg/ml (0.055 mM) apo protein. Visible absorbance was monitored for 10 min after the addition of an equimolar concentration of iron. The iron sources were ferric chloride, ferrous sulfate, and ferric chloride chelated by a 100 mM excess of oxalate. Following the 10-min incubation with ferrous sulfate, sodium bicitarbate was added to a final concentration of 5.5 mM and monitored for an additional 10 min.

Iron density maps were produced from the addition of 0–7 mM sodium pyrophosphate to holo cFbpA or holo nFbpA in 30 mM NaCl, 10 mM Tris-HCl, pH 7.4.

The holo cFbpA crystal grew in space group P21 (a = 94.45, b = 90.70, c = 56.83, and β = 92.39) with two molecules in the asymmetric unit (solvent content of 38%). A comparative model of cFbpA was constructed using the apo FbpA structure from Bordetella pertussis (Protein Data Bank code 1MRP) (14) to generate a model of the holo cFbpA. A molecular replacement solution was obtained using the holo cFbpA coordinates and the program MolRep (20) within the CCP4 suite of programs (21). Interpreta-

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Surprisingly, no density is observed for an anion near the iron-binding site in the 2Fo–Fm map (Fig. 2). The four coordinating Tyr residues are ~1.9 Å from the iron and have B-factors similar to that of the bound iron (1.9 Å). In contrast, His14 forms a weaker interaction with iron (~2.3 Å), and the imidazole ring average B-factor is elevated (20.6 Å²). In addition, the iron-ligand bond is 40° out of the imidazole plane, which intersects the OH atoms of Tyr146 and Tyr203 that are located 3.14 and 2.70 Å from His14 NE2, respectively (see Table SII and Fig. S1).

Surrounding the iron site are networks of charged and polar amino acids (Fig. 1B). Most notably, five Arg residues (Arg13, Arg105, Arg107, Arg140, and Arg183) are within 10 Å of the iron center. The closest noncoordinating group is the guanidinium of Arg105 (4.1 Å). Interestingly, Arg105 in the C-terminal domain is part of a relay of buried salt bridges around one side of

Homologs confirm that cFbpA represents a new class of FbpA and suggest a unique function in iron uptake.

Phylogenetic Analyses—Protein sequences homologous to the FbpA from H. influenzae (YP 001576901, B. pertussis Tomahoma I (NP 880337.1), and C. jejuni NCTC 11168 (NP 261385.1) were identified in the nr database with the BlastP (27) portal at the National Institutes of Health (www.ncbi.nlm.nih.gov/BLAST/). FbpA homologs were selected from the 100 best hits from each BlastP search such that the sequences shared 75% identity or less, exhibited an E value of 10⁻²⁰ or less, and were annotated to identified prokaryotes. Protein sequences were aligned with ClustalW (28) using a gap opening penalty of 15 and a BLOSUM series matrix, followed by the generation of a neighbor-
**FIG. 1.** The crystal structure of cFbpA and comparison of the iron site to hFbpA and mFbpA. In all of the panels, the backbone of the N- and C-terminal domains are in light pink and gray, respectively. Yellow residues are conserved among most FbpAs with few exceptions. Dark pink residues are conserved among most Class I and III FbpAs. Dark purple residues are conserved generally among Classes II, III, and IV FbpAs. Cyan residues are conserved with few exceptions within their respective classes. Synergistic anions and a nonconserved residue are gray, and functionally conserved residues are tan. H-bonds and ligand bonds to the iron (red-pink sphere) are indicated by dotted and solid lines, respectively. The images were generated using MolScript (47) and Raster3D (48).

A, the overall structure of cFbpA with the iron-binding site. B, cFbpA (Class III) iron-binding site. The orange residue is conserved in Class II FbpAs. C, hFbpA (Class I) iron-binding site. The blue sphere is a water molecule, and green residues are conserved functionally in Class I FbpAs. D, the mFbpA (Class II) iron-binding site.

**FIG. 2.** $2F_o - F_c$ representative electron density of the cFbpA chain A iron-binding site, contoured at 0.8 o. Tyrosines and water are dark and light blue, respectively. Histidine is purple, arginine is yellow, and iron is represented by a red sphere. The image was generated using MolScript (47) with changes by R. Esnouf and Raster3D (48).
the iron site that includes the N-terminal domain residues Glu275, Arg201, and Asp18. In addition to connecting the N- and C-terminal domains, Arg201 lies within the hinge region. The N- and C-terminal domains are also connected through a H-bond interaction involving Asn257, also located in the hinge region but on the opposite strand, and Asp68 of the N-terminal domain. A third charge network involving Arg440, Asp902, and Arg107 is found solely in the C-terminal domain. In addition, two C-terminal domain residues form H-bonds with the OH atoms of the Tyr iron ligands (the guanidinium of Arg205 with Tyr15 and the side chain amide of Asn206 with Tyr202).

Iron Binding—The addition of iron to apo cFbpA gives an absorbance maximum at 440 nm, indicative of a ferric-tyrosine interaction (31). Interestingly, the addition of free ferrous iron or ferric iron chelated by oxalate results in significantly higher absorbance readings as compared with free ferric ions (Fig. 3A). Further addition of excess carbonate to the protein sample saturated with ferrous ions does not alter the absorbance reading.

Competition experiments with the iron chelator pyrophosphate were used to define the affinity of cFbpA for iron (Fig. 3B). Increasing concentrations of pyrophosphate blue shifts and decreases the visible absorbance of both holo cFbpA and holo nFbpA. For holo cFbpA, a chelator:protein ratio of 2:1 reduces the initial absorbance reading to half. A ratio of 20:1 is needed for holo nFbpA, indicating that the iron binding constant of cFbpA is likely within an order of magnitude of nFbpA.

Sequence Analyses—The FbpA homologs found in the sequence data base are derived from diverse prokaryotes including proteobacteria, cyanobacteria, Gram-positive bacteria, and archaea. The tree generated by TREE-PUZZLE divides the sequences into eight clusters (Fig. 4, see also Fig. S2). Six classes of FbpAs were defined on the basis of these eight clusters as well as the conservation of residues known to coordinate iron from the FbpA crystal structures. The clusters correspond to individual classes, except for Classes II and III, which are composed of two clusters each. Classes I, II, and III contain more than nine members each and include the structurally characterized nFbpA, mFbpA, and cFbpA, respectively. Classes IV–VI contain three members each and are not yet structurally characterized. Class IV contains FbpAs from environmental bacteria, whereas Class V and VI include FbpA homologs from prokaryotes that are nonpathogens as well as extremophiles and cyanobacteria.

The class definitions are also consistent with observed insertions and deletions in the multiple sequence alignment (Fig. 5). Sequences in Classes I, V, and VI have small deletions near alignment position 179, and only in Class I are two amino acids deleted. Furthermore, Class V has a three-amino acid insertion relative to Classes I and VI near alignment position 235. Classes II and III are distinguished clearly from each other and all other FbpAs by single deletions at alignment position 39 and 214, respectively. Class IV can be distinguished from Classes II and III by a large deletion near alignment position 212.

Only two of the residues known to coordinate iron, Tyr202 and Tyr203 in cFbpA (Fig. 1B) corresponding to alignment positions 204 and 205 (Fig. 5), are conserved in all except one FbpA. The exception is the Class V FbpA from Synechocystis sp. PCC 6803, which appears to have diverged significantly from the family. A third Tyr coordinated to iron in C. jejuni (Tyr146) is conserved in half of the classes (Class II, III, and IV). The fourth iron ligand in cFbpA, Tyr15, is observed only in Class III and is conserved in most of these FbpAs. The remaining ligand, His14, is conserved in both Class I and most of Class III FbpAs.

**DISCUSSION**

The obstacles that organisms face in the acquisition of ferric iron, because of its low solubility and reduction potential, are substantial. Consequently, iron binding and transport proteins bind iron with high affinity. The first picture by x-ray crystallography of a high affinity iron transport protein was of the mammalian transferrin-lactoferrin family (15). The transferrins show iron bound in an octahedral geometry not only by the protein ligands (two Tyr residues, a His, and an Asp) but also by carbonate, a synergistic anion for iron binding. The crystal structures of bacterial iron transport proteins, hFbpA, nFbpA, and mFbpA, also show iron bound using a combination of protein ligands and synergistic anions (one His, one Glu, two Tyr residues, and one phosphate for hFbpA and nFbpA; three Tyr residues and one carbonate for mFbpA). Interestingly, the iron ligands in these bacterial FbpAs are not spatially equivalent to that of transferrin (14). Also, the carbonate-binding site in mFbpA is displaced relative to the anion-binding sites of hFbpA, nFbpA, and transferrin. Nevertheless, these structures suggest an apparent requirement for a synergistic anion in iron transport proteins. Surprisingly, the cFbpA structure in the closed conformation reveals unprecedented iron coordination...
FIG. 4. Unrooted tree of FbpA sequences generated with TREE-PUZZLE (30). Six classes are indicated. H.in, H. influenzae R2866 gi42632152; N.go, Neisseria gonorrhoeae gi1099687; P.ae2, Pseudomonas aeruginosa PA01 gi15599882; P.pu, Pseudomonas putida KT2440 gi26991560; P.fl2, Pseudomonas fluorescens PFO-1 gi23058165; B.fa, Burkholderia fungorum gi22989044; P.fl1, P. fluorescens PFO-1 gi23060872; R.xy, Rubrobacter xylanophilus DSM 9941 gi45546990; M.lo, Mesorhizobium loti MAFF303099 gi13473018; N.sp, Nostoc sp. PCC 7120 gi17228877; G.vi, Gloeobacter violaceus gi37520583; T.er, Trichodesmium erythraeum IMS101 gi23039548; T.el, Thermosynechococcus elongatus BP-1 gi22298056; S.sp, Synechococcus sp. PCC 6301 gi2125893; B.pe, B. pertussis Tohama I gi33592693; A.tu2, Agrobacterium tumefaciens gi15887756; R.pa, Rhodopseudomonas palustris CGA009 gi39937212; A.tu1, A. tumefaciens gi15887755; S.me, Sinorhizobium meliloti gi15964482; V.pa, Vibrio parahaemolyticus RIMD 2210633 gi28899265; P.muc, Pasteurella multocida gi15601916; M.ha, M. hemolytica gi3978164; R.sp, Rhodobacter sphaeroides gi22959567; S.on, Shewanella oneidensis MR-1 gi24372333; R.ru, Rhodospirillum rubrum gi22967044; S.sp2, Synechocystis sp. PCC 6803 gi16329434; E.ch, E. chaffeensis gi4894577; C.vi, Chromatium violaceum ATCC 12472 gi34497436; C.je, C. jejuni subsp. jejuni NCTC 11168 gi15791562; W.pi, Wolbachia pipientis wMel gi42520728; B.eu, Brucella suis 1330 gi23500440; C.pe, C. perfringens gi18309420; H.sp, Halobacterium sp. NRC-1 gi15790051; B.ha, Bacillus halodurans gi15613076; P.ae1, P. aeruginosa PA01 gi15600410; N.eu, Nitrosporosomas europaen ATCC 19718 gi30249052; S.sp3, Synechocystis sp. PCC 6803 gi16330556; M.gr, M. Gryphiusdaldense gi33945217; S.sp1, Synechocystis sp. PCC 6803 gi16331793; S.el, Synechococcus elongatus PCC 7942 gi45512924 (this record has since been replaced with gi46129860); S.ma, S. marcescens gi134455. The tree was displayed with TreeView version 1.6.6 (49).
incorporating only protein ligands. The unique iron-binding mechanism in cFbpA establishes a new class of FbpAs, Class III.

The closed conformation of the crystal structure of iron-loaded cFbpA is defined in comparison with the holo hFbpA structure. Superimposition of 125 CAs from the cFbpA structure, representing the core of both domains, onto the equivalent CAs from the hFbpA structure yields a root mean square deviation of 0.88 Å. In contrast, similar superimposition of both domains of the cFbpA structure with the apo hFbpA structure (Protein Data Bank code 1D9V) is not possible because of a 21° relative domain rotation about the apo hFbpA hinge region, resulting in an open conformation (32).

The unexpected result arising from the cFbpA structure is that iron is bound without the need for a synergistic anion in the closed conformation. Instead iron is coordinated solely by five protein derived ligands (His and four Tyr residues). Inspection of the absent site within the distorted octahedral geometry shows that anion access to this position is sterically hindered by the phenolate rings of Tyr15, Tyr202, and Tyr203 (Fig. 2).

The lack of a synergistic anion is surprising because these anions are thought to play critical roles in the initiation of iron binding and release in iron transport proteins. Carbonate is synergistic for iron binding to transferrin (33), and a low pH crystal structure suggests a role for bound carbonate in the initiation of iron release (34). In Class I FbpAs (hFbpA and mFbpA), phosphate and the two conserved Tyr residues form an iron half-site that is proposed to bind iron before forming a closed holo structure (32). Also, phosphate is necessary for the formation of the closed conformation (26). In mFbpA (Class II), three C-terminal Tyr residues form the analogous half-site, eliminating the need for an anion (phosphate) at this position (35); however, a carbonate anion is observed in the closed holo structure (Protein Data Bank code 1S10) and is the only additional iron ligand (Fig. 1D) (17).

Although a synergistic anion is not observed in the holo cFbpA structure, a combination of iron site residues conserved with either hFbpA or mFbpA may substitute for the functions of a synergistic anion. The presence of Tyr146 suggests a similar preordered half-site formed from three C-terminal Tyr ligands (Fig. 1, A and B). As in hFbpA, the conserved N-terminal His14 (along with Tyr15 of cFbpA) creates a direct iron bridged link between the N- and C-terminal domains. In cFbpA, the near neutral $pK_a$ of His and observed weaker interaction of His14 with iron in the crystal structure suggests a role for this residue in mediating iron release, similar to the role of carbonate in transferrin.

That the anion is superfluous is further emphasized by spectroscopic observations that a synergistic anion is not required for high affinity iron binding to cFbpA (Fig. 3). In addition, recombinant cFbpA is isolated from E. coli loaded with iron. These observations suggest a function for cFbpA as a component of a high affinity iron transport system in C. jejuni. However, the cFbpABC transport system likely functions differently compared with that from Neisseria because C. jejuni is unable to grow on the characterized iron sources typical of this system, transferrin and lactoferrin (4). C. jejuni can use ferrous iron, but disruption of $feoB$ in C. jejuni, a putative ferrous uptake system characterized in Helicobacter pylori and E. coli, did not diminish $^{55}\text{Fe}^{2+}$ uptake compared with the wild-type C. jejuni (36). Binding studies show a preference by cFbpA for ferrous iron as opposed to the free ferric form (Fig. 3A), suggesting that cFbpA may constitute a key component of a ferrous uptake system in C. jejuni. In addition, cFbpA may be involved in the acquisition of the ferric form bound to biological chelators such as oxalate.

Within the cluster containing cFbpA (Fig. 4), four of five sequences are from pathogenic bacteria proposed to have an intracellular stage within the host. Members of this cluster include C. jejuni and Clostridium perfringens, for which the severity of infection is correlated with bacterial invasion (37, 38). C. jejuni is proposed to have an intracellular stage during invasion into the epithelium of the gut (39). C. perfringens, a Gram-positive anaerobe, is the causative agent of gas gangrene and has extensive invasive capacity (40). C. perfringens is proposed to exist within the cytoplasm of macrophages in the

FIG. 5. Representative alignment of a subset of FbpA sequences from Classes I to VI. The abbreviation for each sequence is given in Fig. 4. The class designation is indicated in parentheses. Black shading indicates absolutely conserved residues, and gray shading indicates similar residues. The alignment was viewed using BioEdit (29).
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initial stage of infection (38). The remaining members are from *Ehrlichia chaffeensis* and *Wolbachia pipientis*, which are obligate endocellular symbionts. The unique structural and functional properties of FbpA suggest a novel role for these FbpAs in iron uptake from within a host intracellular environment.

Ligand conservation is also observed within each of the different classes. Phylogenetic analyses of the Class III FbpAs show that the ligands are mainly conserved with few exceptions. One exception includes the FbpA homolog from *Magnetospirillum gryphiswaldense* that lacks the corresponding His<sup>14</sup> and Tyr<sup>15</sup> ligands because of a truncated N terminus. Also, in the FbpAs from *Wolbachia* and *E. chaffeensis*, the residues aligned with His<sup>14</sup> and Tyr<sup>15</sup> of FbpA are Lys and Glu, indicating some change in iron ligation. The iron ligands observed within the crystal structures of Classes I and II are conserved absolutely among the sequences of their respective class members. Class IV–VI FbpAs likely coordinate iron differently than within the crystal structures of Classes I and II are conserved, because three of 41 FbpAs contain a Tyr substitution, this Tyr is present in the FbpA from *Ehrlichia chaffeensis* and *Campylobacter*, which is known to be involved in iron transport (41).

Interestingly, despite the different iron sites between the classes, nearby tertiary interactions are conserved. Comparison of the iron-binding site of cFbpA with the other classes shows that six residues are conserved among all FbpAs with few exceptions, and all except Glu<sup>275</sup> originate from the C-terminal domain (Fig. 1B, *yellow*; see also Table SIII). Only two of these residues are iron ligands (Tyr<sup>202</sup> and Tyr<sup>203</sup>). Conserved residues Arg<sup>105</sup> and Arg<sup>107</sup> are near these tyrosines and likely lower the pK<sub>a</sub> of the phenolates to facilitate iron binding. Glu<sup>275</sup> forms a conserved salt bridge to Arg<sup>105</sup>. In the available crystals structures, Asn<sup>200</sup> seems to assist in iron binding by either forming an H-bond to a Tyr ligand (Class II and III FbpAs) or by interacting with the synergistic anion (Class I FbpAs). Among FbpAs from Classes II, III, and IV, additional cFbpA iron site residues are fully conserved (Arg<sup>13</sup>, Tyr<sup>146</sup>, Asp<sup>182</sup>, and Asn<sup>237</sup>) or mostly conserved (Asp<sup>63</sup> and Asp<sup>140</sup>) (Fig. 1B, *dark purple*). Hydrogen bonding networks that surround the iron site are composed of these residues along with the six residues conserved in most FbpAs. H-bonds linking the N- and C-terminal domains are formed from four of these residues (Arg<sup>13</sup> and Glu<sup>275</sup>, Asp<sup>63</sup> and Asn<sup>237</sup>) and are conserved between these classes. Interestingly, in mFbpA the side chain equivalent to Arg<sup>13</sup> in cFbpA is displaced to form a H-bond to the synergistic carbonate; thus, one of the two H-bonds to the carboxylate group of Glu<sup>275</sup> present in cFbpA is lost in mFbpA (Fig. 1, *B and D*). These interdomain H-bond networks in the closed conformation are also maintained in Class I FbpAs using functionally equivalent residues (Fig. 1C).

Interestingly, some bacteria contain multiple FbpAs (Fig. 4) spanning the different classes. Of the Class III cyanobacterial FbpAs, some are shown to function in iron uptake, whereas others have adapted the iron binding function for alternative purposes. For example, *Synechocystis* sp. PPC 6803 contains three FbpAs, also known as IdiA homologs, two of which are members of Class III (S.sp1 and S.sp2), and one is in Class V (S.sp3). The best characterized is S.sp1 (annotated in the genome sequence as srl1295), which is cytoplasmic and is involved in protecting photosystem II during iron limitation and oxidative stress (42). Although the exact mechanism by which S.sp1 protects photosystem II is not known (42), the iron binding properties are similar to other FbpAs (43). In contrast, S.sp2 (srl0513) is secreted into the periplasm (42, 44) and is likely a component of an iron uptake system (45). The sequences of both S.sp1 and S.sp2 have iron ligands conserved with those of cFbpA, suggesting similar iron coordination without a synergistic anion and mechanism of iron binding and release.

The conservation of the iron site between Class III FbpAs from *Campylobacter* and the cyanobacteria offers precedence for an evolutionary model whereby iron binding was present before the incorporation of various synergistic anion-binding sites in other FbpAs. Cyanobacteria are believed to be the first oxygen-evolving phototrophic organisms and may therefore be primarily responsible for the global conversion from an anoxic to oxic atmosphere (46). These microorganisms may have been the first to adapt to a reduced bioavailability of iron because of the local oxidation of ferrous to ferric ions (42). Thus, cyanobacteria may have developed one of the first iron uptake systems linking ferrous and ferric uptake, a component of which may have resembled cFbpA. Because early microorganisms exchanged genetic material through extensive lateral gene transfer (46), this early iron uptake system may have been readily acquired and modified by other prokaryotes. In contrast, a previous model proposed that FbpAs shared a common anion binding ancestor (14). Our model for FbpA evolution, involving divergence from an anion-independent iron-binding protein ancestor, is more parsimonious.

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