Bacillus cereus iron uptake protein fishes out an unstable ferric citrate trimer

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Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved September 4, 2012 (received for review June 29, 2012)

Citrate is a common biomolecule that chelates Fe(III). Many bacteria and plants use ferric citrate to fulfill their nutritional requirement for iron. Only the *Escherichia coli* ferric citrate outer-membrane transport protein FecA has been characterized; little is known about other ferric citrate-binding proteins. Here we report a unique siderophore-binding protein from the Gram-positive pathogenic bacterium *Bacillus cereus* that binds multinuclear ferric citrate complexes. We have demonstrated that *B. cereus* ATCC 14579 takes up ⁵⁵Fe radiolabeled ferric citrate and that a protein, BC_3466 [renamed FctC (ferric citrate-binding protein C)], binds ferric citrate. The dissociation constant (K_d) of FctC at pH 7.4 with ferric citrate (molar ratio 1:50) is 2.6 nM. This is the tightest binding observed of any *B. cereus* siderophore-binding protein. Nano electrospray ionization–mass spectrometry (nano ESI-MS) analysis of FctC and ferric citrate complexes or citrate alone show that FctC binds diferric di-citrate, and triferric tricitrate, but does not bind ferric di-citrate, ferric monocitrate, or citrate alone. Significantly, the protein selectively binds triferric tricitrate even though this species is naturally present at very low equilibrium concentrations.

Iron is an abundant element, yet free Fe(III) is limited to 10^{-18} M at neutral pH due to the low solubility of iron(III) hydroxides, the predominant species in aerobic conditions (1). Because bacteria require a minimal internal total iron concentration near micromolar (2), they produce low-molecular-weight ligands, siderophores, that chelate iron with high affinity. The bacteria export apo-siderophores (iron-free siderophores) and import iron-chelated siderophores (Fe-siderophores) using selective siderophore-binding proteins and transporters that enable the bacteria to effectively acquire iron from the surrounding environment (1).

Many bacteria produce complex siderophores, such as enterobactin (3) (Fig. S1A) or petrobactin (4) (Fig. S1B), that require specialized proteins to transport and use the Fe-siderophore. The specificity of such systems gives the siderophore-producing species an advantage over neighboring bacteria that do not have the required proteins to acquire the Fe-siderophore. Moreover, some pathogenic bacteria further modify the siderophore that they produce to ensure that no other pathogens or animal hosts can intercept the Fe-siderophore and use the iron payload. For example, *Salmonella* glycosylates enterobactin, making salmochelin, a stealth siderophore (5) (Fig. S1C), that can sequester iron and avoid human siderocalin, an antibacterial protein of the innate immune system (6). The variability of siderophore structures requires that bacteria must manufacture several Fe-siderophore transport systems to acquire the iron made available by the corresponding siderophores. For example, the *Bacillus cereus* group (*B. cereus*, *B. anthracis*, and *B. thuringiensis*) produces at least 10 siderophore-binding proteins to recognize exogenous siderophores (7), although these bacteria produce only bacillibactin and/or petrobactin (7).

The small molecule citrate, although not a powerful or distinctive chelator, can serve as an iron-delivery agent for several bacteria. Citrate is produced by many species via the tricarboxylic acid cycle (8). Moreover, citrate is used as a chelator of Fe(III) not only by bacteria but also by plants (9, 10), and many ferric citrate complexes are found in plant xylem sap (11). Several

siderophores contain a citrate backbone, such as the stealth siderophore petrobactin (12), produced by the *B. cereus* group (7) and *Marinobacter aquaeolei* (13); staphyloferrin A (Fig. S1D), produced by *Staphylococcus aureus* (14); and rhizoferrin (Fig. S1E), produced by the fungus *Rhizopus microsporus* var. *rhizopodiformis* (15). The carboxyl and hydroxyl groups of the citrate backbone of these siderophores coordinate to ferric ion. Thus, the use of citrate and citrate-derivative siderophores as ferric chelators for iron(III) uptake is common in many organisms.

The composition of ferric citrate solutions is complex because multinuclear metal complexes can form (16, 17). Fig. 1 shows the structural formulas of different ferric citrate coordination complexes found in aqueous conditions, including ferric monocitrate (FeCit) (ML), ferric di-citrate (FeCit₂) (ML₂), diferric di-citrate (Fe₂Cit₂) (M₂L₂), and triferric tricitrate (Fe₃Cit₃) (M₃L₃) (16). Structural information obtained in the solid state is available for the ML₂ and M₂L₂ species (17, 18), as well as for more complex systems such as nona-iron(III) citrate (19). However, the Fe₃Cit₃ structure is not known. A structure of Fe₃Cit₃ has been proposed (17), and the [Fe₃O(Cit)₃]^{2−} species has been identified in aqueous solutions (16, 17) and in plant xylem sap (11).

With the exception of the Gram-negative bacterium *Escherichia coli* FecA complexes of ferric citrate within binding proteins have not been structurally analyzed. *E. coli* uses an outer-membrane protein, FecA, to import ferric citrate into the periplasm. The periplasmic ferric citrate-binding protein FecB, inner membrane transporters FecC and FecD, and the ATPase FecE, then transport the ferric citrate into the cytoplasm (20). A structure of FecA shows that it binds either two molecules of iron-free citrate or the iron dimer Fe₂Cit₂ (21). Recently, Gomez et al. showed that protein-tyrosine-phosphatases (PTPs) are regulated by mononuclear iron di-citrate (FeCit₂), and a complex of FeCit₂ and macrophage PTP SHP-1 (Protein Data Bank code: 1GWZ) was predicted by computational molecular docking (22).

Although the ferric citrate-uptake machinery FecABCDE is well described and is conserved in other Gram-negative bacteria such as *Pseudomonas* and *Shigella*, less is known about Gram-positive ferric citrate transport systems. The Gram-positive bacterium, *Bacillus subtilis*, possesses ferric citrate-uptake machinery in the form of YfmC (ferric citrate-binding protein), YfmD and YfmE (membrane transporters), and YfmF (ATPase) (23).

In this paper, we investigate a unique ferric citrate-binding protein in the pathogenic bacterium *Bacillus cereus* and characterize the bound ferric citrate substrates. The *B. cereus* group (*B. cereus*, *B. anthracis*, and *B. thuringiensis*) does not have proteins

Author contributions: T.F. and K.N.R. designed research; T.F., A.K.S., R.N., and Z.Z. performed research; T.F., A.K.S., B.E.A., R.N., and U.N.A. analyzed data; and T.F., A.K.S., B.E.A., and K.N.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1210131109/-DCSupplemental.

Fig. 1. Proposed structures of ferric citrate complexes in neutral aqueous solution. Citrate coordinates Fe(III) as FeCit (ML) (A), FeCit_2 (ML_2) (B), Fe_2Cit_2 (M_2L_2) (C), and Fe_3Cit_3 (M_3L_3) (D). Structures B and C are 2D bond-line drawings generated from the crystal structures of Gautier-Luneau et al. (17). Structure D is a 2D model proposed by Gautier-Luneau et al. (17). The Gautier-Luneau et al. model shows dangling citrate carboxylates uncoordinated to Fe(III), which we suggest does not occur (Fig. 5).

similar to the ferric citrate uptake proteins of *B. subtilis*, YfmCDEF. Using a ^{55}Fe -citrate radiotracing experiment, protein fluorescence quenching experiments, and nano ESI-MS analyses, we demonstrate that *B. cereus* strain ATCC 14579 takes up ferric citrate and that ferric citrate-binding protein C (FctC) (BC_3466) is selective for the multinuclear ferric citrate species Fe_3Cit_3 . It is the tightest binding observed of any *B. cereus* siderophore-binding proteins, including FeuA, FpuA, and FatB (24), and we suggest that selecting the unstable trimer is part of the iron specificity of the uptake system.

Results

***B. cereus* ATCC 14579 Imports the Ferric Citrate Complex.** It is known that several bacterial siderophore-binding proteins such as *E. coli* FecA and FecB bind ferric citrate (25, 26) and that some bacteria such as *E. coli* and *Mycobacterium smegmatis* take up ferric citrate as if it were a Fe-siderophore (20, 27). Within the *Bacillus* species, YfmC has been identified in vivo as a ferric citrate-binding protein of *B. subtilis* (23), and we suspected that *B. cereus* also has a ferric citrate-binding protein. To confirm this, a ^{55}Fe -citrate uptake assay was performed in iron-limited growth media. As shown in Fig. S2, *B. cereus* was able to import ^{55}Fe -citrate (open circles). After 2 min of incubation, the addition of cold Fe-citrate to the media resulted in inhibition of ^{55}Fe -citrate uptake (closed squares in Fig. S2). This result clearly demonstrates that *B. cereus* ATCC 14579 possesses ferric citrate uptake machinery.

FctC (BC_3466) in *B. cereus* ATCC 14579 Binds Ferric Citrate. Hotta et al. reported that *B. cereus* ATCC 14579 has at least 10 genes that encode putative siderophore-binding proteins (7). Five genes have been identified, including those which encode a

desferrioxamine B/ferrichrome-binding protein (YxeB), a schizokinen-binding protein (YfiY), an enterobactin/bacillibactin-binding protein (FeuA), and petrobactin-binding proteins (FpuA and FatB) (24). The other less known but predicted siderophore-binding proteins of *B. cereus* (BC_2208, BC_3466, BC_4363, BC_4416, and BC_5380) were isolated and purified, and their affinities for ferric citrate were tested by fluorescence quenching (FQ) assays. Proteins BC_4363, BC_4416, and BC_5380 were not quenched by ferric citrate (Fig. S3 A–C), indicating they are not ferric citrate-binding proteins. The fluorescence of BC_2208 was quenched by addition of TBS buffer (25 mM Tris-HCl, 8 g/L NaCl, 0.2 g/L KCl) only (Fig. S3D), making it difficult to assess the binding affinity of BC_2208 for ferric citrate. The fluorescence of BC_3466 (FctC) was quenched upon addition of ferric citrate (1:1 molar ratio), although the quenching was weak (closed triangles in Fig. 2A). The binding ability of FctC for noncitrate ferric siderophores (Fig. S1) was also assessed by FQ experiments. No significant FQ was detected for these ferric siderophores (Fig. S4A).

Several citrate complexes of Fe(III) have been described (16, 17). Silva et al. characterized the ferric citrate structures in aqueous solution as $[\text{Fe}_2(\text{Cit})_2]^{2-}$ (M_2L_2), $[\text{Fe}_3(\text{Cit})_3]^{3-}$ (M_3L_3), $[\text{Fe}(\text{Cit})_2]^{3-}$ (ML_2), and $[\text{Fe}(\text{HCit})]^0$ (MLH) (16) (Fig. 1). Because the relative concentrations of these species change with pH and concentrations, FQ experiments were performed at pH 7.4 with a solution of several ferric citrate molar ratios. As seen in Fig. 2A, FctC was significantly quenched by a solution of Fe:citrate with molar ratio of 1:50 (closed circles), although the quenching of FctC with Fe:citrate molar ratios of 1:1, 1:2, 1:5, and 1:10 was weak [dissociation constants (K_d s): 500–900 nM]. The protein showed no FQ for iron-free citrate (crosses in Fig. 2A), indicating that citrate alone is not bound by FctC.

Because Fig. 2A shows that the FQ of FctC is dependent on the molar ratios of Fe(III) and citrate, the affinity of FctC for ferric citrate was investigated in FQ experiments with several molar ratios of Fe(III) and citrate (Fe:citrate = 1:1, 1:10, 1:25, 1:50, 1:100, and 1:200) and at several pH values (pH = 3.5, 4.5, 5.5, 8.0, and 8.5). In basic buffer conditions, FctC was quenched with only higher citrate:Fe molar ratios at pH 8.0 (Fe:citrate = 1:50, 1:100, 1:200) (Fig. 2B), and at pH 8.5 the FQ was less prominent (Fig. S4B). However, in acidic buffer conditions FctC was significantly quenched with several citrate molar ratios at pH 5.5 and 4.5 (Fig. 2 C and D, respectively). At pH 3.5, FctC showed little FQ upon addition of ferric citrate (Fig. S4C). Increasing the pH of the solution to 4.3 with NaOH resulted in a binding event indicated by a 50% decrease in fluorescence (Fig. S4D). These results indicate that the protein remains intact at pH 3.5, but that there are no suitable ferric citrate species available for binding.

Calculation of the K_d of FctC for Ferric Citrate. FctC was maximally quenched by ferric citrate with a ferric:citrate molar ratio of 1:50 at pH 7.4 (closed circles in Fig. 2A), which was matched by several quenching curves at pH 4.5 (Fig. 2D). However, the K_d s could not be calculated because the data set could not be fit without excessive error. A K_d was calculated for the protein-substrate interaction of FctC using a lower protein concentration (Materials and Methods) with ferric:citrate (Fe:citrate molar ratio 1:50) at pH 7.4. The fluorescence intensity quenching curve (Fig. S5) was fit by Hyperquad (28) to give a K_d of 2.60 nM ($\text{SD} = 0.04$). Such a ferric citrate solution (Fe: Cit molar ratio of 1:50 and pH = 7.4) contains a mixture of ferric citrate species (Fe_2Cit_2 , FeCit_2 , etc.). Thus, the calculated K_d for this system represents an upper limit of the affinity that FctC has for all species in solution.

Ferric Citrate Speciation. The fluorescence of FctC decreases by different amounts depending on the composition (Fe:citrate molar

Fig. 2. FctC FQ experiments. FQ of FctC with several ferric citrate molar ratios was measured at pH 7.4 (A), pH 8.0 (B), pH 5.5 (C), and pH 4.5 (D). The ferric: citrate molar ratios used in this study were 1:1 (closed triangles), 1:2 (open triangles), 1:5 (open diamonds), 1:10 (closed diamonds), 1:25 (closed squares), 1:50 (closed circles), 1:100 (open circles), and 1:200 (open squares). Crosses represent citrate alone and the concentrations (μM) are shown in the lower x axis in A. The calculated K_d s of FctC with ferric: citrate molar ratios of 1:1, 1:2, 1:5, and 1:10 at pH 7.4 and with ferric: citrate molar ratios of 1:1, 1:10, and 1:25 at pH 8.0 were 500–900 nM and $>1 \mu\text{M}$, respectively.

ratio and pH) of the titrant. Speciation diagrams using known thermodynamic data were generated with Hyperquad Simulation and Speciation (HySS) (29) to predict the relative formation of ferric citrate species in solution under specific conditions. The calculation was performed assuming a concentration of $1.25 \mu\text{M}$ of Fe(III) and either 62.5 or 125 μM of citrate (Fig. 3 A and B). The Fe(III) concentration of the calculation corresponds to the final conditions of the FQ experiment of FctC [$1.25 \mu\text{M}$ Fe(III) in Fig. 2 and Fig. S4 B and C]. From Fig. 3 A and B, solutions with $1.25 \mu\text{M}$ of Fe(III) and 62.5 or 125 μM of citrate have the M_2L_2 ($[\text{Fe}_2(\text{Cit})_2]^{2-}$) as the main species and M_3L_3 ($[\text{Fe}_3(\text{Cit})_3]^{3-}$) as the minor species at pH 4.5 and 5.5. Because FctC was significantly quenched under these two pH conditions (Fig. 2 C and D), this suggests that FctC binds M_2L_2 and/or M_3L_3 . At pH 3.5, MLH ($[\text{Fe}(\text{HCit})]$) is the predominant species in solution, and the pH 8.0 and 8.5 solutions favor ML_2 ($[\text{Fe}(\text{Cit})_2]^{5-}$) (Fig. 3 A and B). The magnitude of FQ of FctC in pH 8.0 solution was decreased compared with the quenching at pH 4.5 and 5.5 (Fig. 2 B–D). Ferric citrate solutions at pH 8.5 contain mostly ML_2 species and $<1\%$ M_2L_2 and M_3L_3 (Fig. 3 A and B) as determined by speciation calculations. This solution was then used in FQ assay to

assess the affinity of FctC for ML_2 . The results (Fig. S4B) suggest that FctC does not have a significant affinity for ML_2 .

Nano ESI-MS Analysis Shows That FctC Binds Fe_3Cit_3 and Fe_2Cit_2 . Complexes of FctC and ferric citrate were analyzed by nano ESI-MS at pH 6.9 with the ferric: citrate molar ratios of 1:1 and 1:10. The speciation diagram by HySS in these conditions predicts that solutions with a ferric: citrate molar ratio of 1:1 predominantly form the Fe_3Cit_3 (M_3L_3) and Fe_2Cit_2 (M_2L_2) species (Fig. 3C), and solutions with a ferric: citrate molar ratio of 1:10 form Fe_2Cit_2 and FeCit_2 (ML_2) as the main species and Fe_3Cit_3 and FeHCit_2 (ML_2H) as the minor species (Fig. 3D). At pH 6.9, FctC was observed by nano ESI-MS with the most abundant peaks corresponding to the +15 and +16 charge states (peaks A15 and A16 in Fig. S6A). Data analysis by MassLynx software resulted in a molecular weight of 33,939.5 Da, which is in a good agreement with the theoretical value of 33,941.7 Da (Table S1). Nano ESI-MS confirmed the formation of two noncovalent complexes: FctC with Fe_3Cit_3 (peaks B15 and 16 in Fig. S6 B and C) and Fe_2Cit_2 (peaks C15 and C16 in Fig. S6C). Fig. S6 B and C shows mass spectra corresponding to the charged states +15 and +16 detected for both complexes. The molecular weights determined from the experimental data are 34,677.9 Da for the FctC– Fe_3Cit_3 complex (theoretical value 34,676.5 Da) and 34,433.0 Da for the FctC– Fe_2Cit_2 complex (theoretical value

Fig. 3. Speciation diagrams of ferric citrate. A speciation diagram was generated with HySS as described in SI Materials and Methods. (A and B) The calculation was performed with ferric: citrate molar ratios of 1:50 (1.25 μM : 62.5 μM , A) and 1:100 (1.25 μM : 125 μM , B). The Fe(III) and citrate concentrations are derived from the final conditions of the FQ experiments (1.25 μM Fe concentration in Fig. 2 and Fig. S4 B and C). (C and D) The calculation was performed with ferric: citrate molar ratios of 1:1 (50 μM : 50 μM , C) and 1:10 (50 μM : 500 μM , D). The Fe(III) and citrate concentrations are the same as those in the nano ESI-MS analysis conditions in Fig. 4 and in Figs. S6 A–C and S7C. Fe_2L_2 , $[\text{Fe}_2(\text{Cit})_2]^{2-}$; Fe_3L_3 , $[\text{Fe}_3(\text{Cit})_3]^{3-}$; FeL_2 , $[\text{Fe}(\text{Cit})_2]^{5-}$; FeLH , $[\text{Fe}(\text{HCit})]$; FeL_2H , $[\text{Fe}(\text{HCit})_2]^{4-}$; FeH_2 , $[\text{Fe}(\text{OH})_2]^{+}$.

34,431.6 Da), respectively (Table S1). These results show that FctC selectively binds Fe_3Cit_3 and Fe_2Cit_2 .

Yue et al. reported that *E. coli* FecA protein binds not only Fe_2Cit_2 but also two molecules of citrate (21). In a sample of FctC and 50 μM citrate mixture, no complex was detected (only FctC was detected as peaks A15 and A16 in Fig. S7A), again indicating that FctC cannot bind iron-free citrate.

FctC did not complex FeCit_2 in a solution at pH 6.9 with ferric: citrate molar ratio 1:10 (Fig. S6C) in which FeCit_2 is a major species (Fig. 3D). A 1:500 Fe: citrate solution is also predicted to have ML_2 as a predominant species (Fig. S8A). Such a solution was mixed with solutions of FctC, and the protein-substrate solution was analyzed by mass spectrometry (nano ESI-MS). The resulting spectrum (Fig. S7B) makes it clear that FctC did not bind FeCit_2 in a ferric: citrate molar ratio of 1:500.

FctC Selectively Binds Fe_3Cit_3 . FctC was quenched by the addition of ferric citrate from pH 4.5–7.4 and was less quenched at pH 8.0 and 8.5 (Fig. 2 and Fig. S4B). To identify the exact ferric citrate species bound by FctC, we made ferric citrate solutions with ferric: citrate molar ratios of 1:1 and 1:10 at pH 5.5 and 8.0, mixed these with solutions of FctC, and analyzed ternary complex formation by nano ESI-MS. At pH 5.5 (Fe: citrate = 1:1 and 1:10) and at pH 8.0 with ferric: citrate molar ratio 1:1, Fe_2Cit_2 and Fe_3Cit_3 are the first and second most abundant species, respectively (Fig. 3 C and D). At pH 8.0 with a ferric: citrate molar ratio of 1:10, FeCit_2 predominates, and Fe_2Cit_2 and Fe_3Cit_3 become less prominent species in solution (Fig. 3D). As shown in Fig. 4 B and C, FctC formed a complex with Fe_3Cit_3 at pH 5.5 even though the solution contains not only Fe_3Cit_3 but also

Fe_2Cit_2 as the predominant species. Moreover, at pH 8.0 with a ferric: citrate molar ratio of 1:1, FctC bound only Fe_3Cit_3 (Fig. 4E). Remarkably, FctC binds only Fe_3Cit_3 at pH 8.0 with a ferric: citrate molar ratio of 1:10 (Fig. 4F) even though the speciation diagrams predict a low concentration of Fe_3Cit_3 (less than 5% formation) (Fig. 3D). Furthermore, FctC bound Fe_3Cit_3 at pH 9.0 (ferric: citrate molar ratio 1:10) (Fig. S7C), even though under these conditions FeCit_2 is the predominant species (Fig. 3D). These results strongly suggest that FctC selectively binds Fe_3Cit_3 and that FeCit_2 is not a suitable target for the protein.

Discussion

Substrate Selectivity of FctC for Ferric Citrate. The ferric citrate complexes FeCit_2 and FeCit are not bound by FctC. Although FeCit_2 is the major species in solution at pH 8.0–9.0 with a ferric: citrate molar ratio of 1:10 (Fe: citrate: 50:500 μM) (Fig. 3D), the nano ESI-MS results at pH 8.0 and 9.0 clearly demonstrate that FctC does not bind FeCit_2 (Fig. 4F and Fig. S7C). Furthermore, FQ experiments of FctC, performed at pH 8.5, where FeCit_2 predominates in solution, show a significant decrease in fluorescence change upon addition of ferric citrate solution. This indicates that FeCit_2 does not bind FctC. Ferric citrate solutions made to optimize the formation of FeCit were then tested for binding to FctC by similar methods. A complex of FctC and FeCit could not be detected by nano ESI-MS. Moreover, the FQ experiment at pH 3.5 showed that there is no suitable ferric citrate substrate for FctC under these conditions (Fig. S4C), even though speciation calculations indicate that the solution contains

Fig. 4. Nano ESI-MS analysis of FctC and ferric citrate complexes at pH 5.5 (A–C) and pH 8.0 (D–F). FctC and ferric citrate complexes were observed with several charge states in positive mode. (A and D) FctC only. (B and E) FctC and ferric citrate (1:1) mixture. (C and F) FctC and ferric citrate (1:10) mixture. Predicted ferric citrate compositions are shown with a speciation diagram in Fig. 3 C and D. Peak A series, FctC protein only; peak B series, FctC and Fe_3Cit_3 complex. Charged states of +15 and +16 were detected, and the numbers correspond to the calculated values in Table S2. The measured molecular weights of FctC and ferric citrate complexes using MassLynx software (Waters) are shown in Table S1.

Molecular Scheme for Fe₃Cit₃. Although a structure for Fe₃Cit₃-oxo has been modeled (11), we propose a structure for Fe₃Cit₃ (Fe₃C₁₈H₁₅O₂₁) that is consistent with the mass spectra results (Fig. 4 and Figs. S6A–C and S7C). Ideas for a structural starting point were inspired by a trialuminum tricitrate-hydroxo crystal structure (30) as well as by a Fe₃L₃ structure [L: *N,N'*-bis(2-pyridylmethyl)-3-aminoacetate in ref. 31] to generate the Fe₃O₃ core (Fig. 5). Citrate ligands were then built around the Fe₃O₃ core using the Gaussian 2009 package. Unlike in the model of Gautier-Luneau et al. (17), all available carboxylate oxygens of citrate are modeled as bound to ferric ion centers because these hard oxygen donors have a strong affinity for Fe(III). The schematic structure was then optimized in the Schrodinger Inc. graphical user interface Maestro (32). This model was minimized by a molecular mechanics approach (Schrodinger Inc. Macro-model package, OPLS 2005 force field; solvent model: water). Our model is of a hypotheticalal iron citrate trimer in solution; we expect that the water ligands would be replaced in the protein-binding pocket by donors from amino acid residues of FctC.

Fig. 5. Schematic model for Fe_3Cit_3 . Each iron center (orange spheres) is pseudo-octahedral, and each has a coordinated water molecule or peptide ligand from the binding pocket of FctC [green atoms (*Left*) and L (*Right*)] to fill the sixth coordination site. Red atoms are oxygen atoms and hydrogen atoms are removed for clarity. The geometric parameters (distances and angles) around iron are shown in [Table S3](#).

Binding Affinity of FctC for Ferric Citrate. The K_d of FctC for ferric citrate determined by FQ experiment was 2.60 nM (Fig. S5). Because the solution in the quenching experiment contains Fe_2Cit_2 and FeCit_2 as major species and Fe_3Cit_3 and $\text{Fe}(\text{HCit})$ as minor species (Fig. S8C) and FctC can bind only Fe_2Cit_2 and Fe_3Cit_3 , the apparent K_d of the protein actually describes the protein affinity for both Fe_2Cit_2 and Fe_3Cit_3 . The calculated K_d for this system represents an upper limit of the affinity that FctC has for all species in solution; because Fe_3Cit_3 is a minor species (less than 5% according to the speciation), the affinity of FctC for the substrate must be higher ($K_d < 2.60$ nM). Using the titration equivalence point (FctC– Fe_3Cit_x complex: 150 nM) (Fig. S5), the calculated affinity of FctC for Fe_3Cit_3 is 0.267 nM. Previously, Zawadzka et al. described that the K_d of FeuA for Fe-bacillibactin, FpuA for Fe-petrobactin, and FatB for Fe-petrobactin is 19, 175, and 127 nM, respectively (24). Moreover, the K_d of the other *B. cereus* siderophore-binding proteins, YxeB for Fe-desferrioxamine and YfiY for Fe-schizokinen, is 18 and 34 nM, respectively (24). Only the K_d of FatB for $[\text{Fe}-(3,4\text{-dihydroxybenzoic acid})_2]^{3-}$ has a similar value (1.2 nM) to the K_d of FctC for ferric citrate (24). The small K_d of the FctC ferric citrate system indicates that FctC strongly binds multinuclear ferric citrate (Fe_2Cit_2 and Fe_3Cit_3).

Unique Ferric Citrate-Binding Protein, FctC, and Its Operon Proteins.

The *fctC* (*BC_3466*) gene makes an operon with *BC_3468* (renamed *fctA*) and *BC_3467* (renamed *fctB*) that encode putative permeases. Because the operon *fctABC* contains no ATPase gene and *B. cereus* ATCC 14579 can uptake ferric citrate (Fig. S2), *B. cereus* must contain the ATPase gene for the machinery of ferric citrate uptake. FctC is conserved in the *B. cereus* group (*B. cereus*, *B. anthracis*, and *B. thuringiensis*), especially *B. cereus* and *B. thuringiensis*. However, only some bacteria, such as *B. mycoides*, *B. pumilus*, and *B. amyloliquefaciens*, have homologous proteins, and the ferric citrate-binding protein YfmC in *B. subtilis* is not similar to FctC. Moreover, the putative permease protein sequences in *B. cereus*, FctA and FctB, are not well conserved except in the *B. cereus* group, *B. mycoides*, *B. pumilus*, and *B. amyloliquefaciens*. The *B. subtilis* YfmCDEF proteins are conserved in Gram-positive and -negative bacteria, including the FecBCDE proteins of *E. coli*. FctC and the predicted permeases constitute a unique ferric citrate uptake system for the *B. cereus* group.

The ferric citrate trimer is fished out of a solution in which it is a minor component. As shown in Fig. S9, the speciation and structures of the Al(III) citrate complexes are significantly different from the Fe(III). We suggest that the recognition of the ferric trimer structure is part of the selective recognition of iron in this uptake system.

Materials and Methods

⁵⁵Fe-Citrate Uptake Assay. ⁵⁵Fe-citrate uptake assays were performed as described previously (24, 33, 34). A detailed protocol is described in *SI Materials and Methods*.

Construction of a Plasmid, pCTF43, for Overexpressing *B. cereus* FctC (BC_3466). The *fctC* gene was inserted into pET28(a) to construct pCTF43. The detailed construction method is described in [SI Materials and Methods](#). The plasmid was used for the overexpression of FctC (aa 21–302).

Overexpression and Purification of FctC. FctC was overexpressed in *E. coli* BL21(DE3) containing pCTF43. A detailed method of purification of FctC is described in [SI Materials and Methods](#).

FQ Experiment. FQ of FctC by the addition of iron-free citrate, ferric citrate, and Fe-siderophores was measured with a Varian Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of $\lambda_{\text{exc}} = 280$ nm, an emission wavelength of $\lambda_{\text{em}} = 320\text{--}370$ nm, excitation and emission slits of

10 and 5 nm, respectively, and photomultiplier tube detector voltages of 735 or 740 V.

To analyze the binding ability of FctC for ferric citrate, solutions of several ferric: citrate molar ratios at several pH values were prepared. The titration of the protein with ferric citrate solution was monitored by fluorescence spectroscopy. During sample preparation and FQ experiments, light exposure of the samples was minimized to avoid generating ferric citrate photoproducts. The detailed protocol is described in *SI Materials and Methods*.

The dissociation constant was calculated from FQ data using Hyperquad. In this experiment, the concentration of FctC was 25 nM in TBS buffer (pH 7.4)/5% (vol/vol) DMSO containing 32 μ g/mL ubiquitin, and the ferric citrate ligand solution had a Fe: citrate molar ratio of 1:50. The data were analyzed with a one-site binding model in Hyperquad (28), which uses a least-squares regression analysis.

Nano ESI-MS. The formation of any ternary species in solution was followed by nano ESI-MS in positive mode (Q-TOF Premier, Waters). One hundred microliters of ferric citrate solution with a Fe: citrate molar ratio of 1:1 or 1:10 (100:100 μ M or 100:1,000 μ M) in 10 mM ammonium acetate (pH 5.5 and 6.9) or ammonium bicarbonate (pH 8.0 and 9.0) buffer was equilibrated for 1.5 h. The ferric citrate solution was mixed with 100 μ L of $\sim$ 100 μ M FctC dialyzed against 10 mM ammonium acetate (pH 5.5 and 6.9) or ammonium bicarbonate (pH 8.0 and 9.0), and the mixture was equilibrated for 20 min. The final concentrations of Fe(III) and citrate in the mixture were either 50 and 50 μ M (1:1 molar ratio) or 50 and 500 μ M (1:10 molar ratio).

To analyze the complex of FctC and ferric citrate at a much higher citrate molar ratio (Fe: citrate: 1:500), 100 μ L of ferric citrate solution with a Fe: citrate molar ratio of 1:500 (0.1:50 μ M) in 10 mM ammonium acetate (pH 6.9) buffer was equilibrated for 1.5 h. The ferric citrate solution was mixed with 100 μ L of $\sim$ 10 μ M FctC dialyzed against 10 mM ammonium acetate (pH 6.9), and

the mixture was equilibrated for 20 min. The final concentrations of Fe(III) and citrate in the mixture were 0.05 and 25 μ M, respectively. During sample preparations and nano ESI-MS experiments, light exposure of the samples was minimized to avoid creating any ferric citrate photoproducts.

For analysis of FctC and citrate alone, 100 μ L of $\sim$ 100 μ M FctC dialyzed against 10 mM ammonium acetate (pH 6.9) and 100 μ L of 100 μ M citrate were mixed, and the mixture was equilibrated for 20 min. The final concentration of citrate in the mixture was 50 μ M.

The solutions of protein and protein-substrate complexes were infused in the nano ESI source with source parameters as follows: capillary voltage: 1.6 kV; sampling cone voltage: 56 V; and source temperature: 80 $^{\circ}$ C. MassLynx software (Waters) was used for data analysis.

Speciation Diagram of Ferric Citrate. Speciation diagrams were generated with HySS (29). The detailed calculation is described in *SI Materials and Methods*.

Triferric Tricitrate Structural Modeling. Fe_3Cit_3 was constructed using the Gaussian 2009 package. We then used the Schrodinger Inc. graphical user interface Maestro (32). The detailed method is described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Professor B. Rowe Byers for kindly providing crude petrobactin extract, Dr. Anna M. Zawadzka for nano ESI-MS analytical advice, and Dr. Chris Andolina for optimizing the fluorometer parameters for these experiments. The authors also thank Dr. Kathy Durkin and Dr. Xinzhen Yang for help with computer modeling, funded through their National Science Foundation Grants (CHE-0233882) and (CHE-0840505). This work is supported by National Institutes of Health Grants AI11744 (to K.N.R.) and 1S10RR022393-01 for the acquisition of the Q-TOF mass spectrometer.

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