Siderophore-controlled Iron Assimilation in the Enterobacterium Erwinia chrysanthemi

EVIDENCE FOR THE INVOLVEMENT OF BACTERIOFERRITIN AND THE Suf IRON-SULFUR CLUSTER ASSEMBLY MACHINERY

Received for publication, October 7, 2008, and in revised form, November 6, 2008 Published, JBC Papers in Press, November 6, 2008, DOI 10.1074/jbc.M807749200

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The intracellular fate of iron acquired by bacteria during siderophore-mediated assimilation is poorly understood. We investigated this question in the pathogenic enterobacterium Erwinia chrysanthemi. This bacterium produces two siderophores, chrysobactin and acromobactin, during plant infection. We analyzed the distribution of iron into cytosolic proteins in bacterial cells supplied with 59Fe-chrysobactin using native gel electrophoresis. A parental strain and mutants deficient in bacterioferritin (bfr), mini-ferritin (dps), ferritin (ftnA), bacterioferredoxin (bfd), or iron-sulfur cluster assembly machinery (sufABCDES) were studied. In the parental strain, we observed two rapidly 59Fe-labeled protein signals identified as bacterioferritin and an iron pool associated to the protein chain-elongation process. In the presence of increased 59Fe-chrysobactin concentrations, we detected mini-ferritin-bound iron. Iron incorporation into bacterioferritin was severely reduced in nonpolar sufA, sufB, sufD, sufS, and sufE mutants but not in a sufC background. Iron recycling from bacterioferritin did not occur in bfd and sufC mutants. Iron depletion caused a loss of aconitase activity, whereas ferric chrysobactin supplementation stimulated the production of active aconitase in parental cells and in bfr and bfd mutants. Aconitase activity in sufA, sufB, sufD, sufS, and sufE mutant strains was 10 times lower than that in parental cells. In the sufC mutant, it was twice as low as that in the parental strain. Defects observed in the mutants were not caused by altered ferric chrysobactin transport. Our data demonstrate a functional link between bacterioferritin, bacterioferredoxin, and the Suf protein machinery resulting in optimal bacterial growth and a balanced distribution of iron between essential metalloproteins.

Iron is necessary for most forms of life, being required for the catalytic activity of essential proteins mediating electron trans-
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a variety of iron-sulfur proteins, whereas those encoded by the suf operon play a role in iron-sulfur cluster biosynthesis under conditions of iron deficiency or oxidative stress (13–15). The iron donation step for cluster assembly in vivo is unknown (16, 17). In eukaryotes, the mitochondrial frataxin protein has been proposed to act as an iron donor for assembly of iron-sulfur clusters (18, 19) and a similar function was assigned to the bacterial homolog CyaY for the lsc machinery, although this still needs to be confirmed (16). The ability of the Suf pathway to function when iron is limiting suggests not only that Suf is involved in iron-sulfur cluster assembly but also that Suf may act as an iron trap.

Ferritins are iron storage proteins that sequester iron in a nonreactive form, protecting the cell from iron-induced toxicity (20, 21). In bacteria, these proteins are present in the same compartment as other iron-requiring proteins falling into three categories: heme-free ferritins (Ftn), found in prokaryotes and eukaryotes; heme-containing bacterioferritins (Bfr), found only in bacteria; and Dps proteins, also called mini-ferritins, present only in prokaryotes (22, 23). Ferritins and bacterioferritins are composed of 24 identical subunits, and Dps proteins contain 12 identical subunits. These subunits assemble to make a spherical protein shell surrounding a central cavity able to hold up to between 2,000 and 3,000 ferric iron atoms for ferritins and 500 atoms for mini-ferritins (21, 24). Ferritins have a binuclear di-iron center constituting the ferroxidase center, which is involved in the oxidation of the ferrous iron (25–28). Ferritins can act as acceptors and donors of ferrous ions, but their precise contribution in bacterial iron metabolism is not well understood.

An appropriate system physiologically relevant and representative of the bacterial world is needed to address these unresolved issues. We have developed E. chrysanthemi (Dickeya dadantii) as a model plant pathogen to investigate the role of iron during infection (29). The genome sequence of this bacterium is available and like many other bacterial species pathogenic to mammals, E. chrysanthemi requires powerful iron transport routes to obtain iron from host tissues. Notably, this bacterium produces two siderophores, chrysobactin and achromobactin, which are important for its virulence (30). Siderophores such as chrysobactin and achromobactin attract iron-containing compounds to the bacterial surface, which is involved in the oxidation of the ferrous iron (31). Bacteriophage infection also reveals the existence of a chrysanthemi (32). The gene is positively controlled by iron and the Fur repressor ftmA, which is involved in the oxidation of the ferrous iron (32, 33). Ferritins are iron storage proteins that sequester iron in a nonreactive form, protecting the cell from iron-induced toxicity (20, 21). In bacteria, these proteins are present in the same compartment as other iron-requiring proteins falling into three categories: heme-free ferritins (Ftn), found in prokaryotes and eukaryotes; heme-containing bacterioferritins (Bfr), found only in bacteria; and Dps proteins, also called mini-ferritins, present only in prokaryotes (22, 23). Ferritins and bacterioferritins are composed of 24 identical subunits, and Dps proteins contain 12 identical subunits. These subunits assemble to make a spherical protein shell surrounding a central cavity able to hold up to between 2,000 and 3,000 ferric iron atoms for ferritins and 500 atoms for mini-ferritins (21, 24). Ferritins have a binuclear di-iron center constituting the ferroxidase center, which is involved in the oxidation of the ferrous iron (25–28). Ferritins can act as acceptors and donors of ferrous ions, but their precise contribution in bacterial iron metabolism is not well understood.

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In this study, we investigated the distribution of iron bound to cytosolic proteins from E. chrysanthemi mutant cells defective in siderophore biosynthesis, supplied with 59Fe via chrysobactin. The addition of iron stimulates growth and leads to global metabolic recovery in iron-deprived cells. We compared mutants deficient in bacterioferritin, bacterioferredoxin, ferritin, mini-ferritin, various Suf proteins, and Fur protein. We demonstrated a functional link between Bfr, Bfd, and the Suf protein machinery, which results in the balanced distribution of iron between essential proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Microbiological Techniques—The bacterial strains and plasmids used are described in supplemental Table S1. The cbsE-1, cbsC-1, acsA-37, acsF-::Ω and dps-Ω mutations were introduced into the appropriate bfr, ftmA, suf, and fur deficient mutants by transduction using phage φEC2 as described previously (35). The rich media used were L broth and L agar (36). Tris medium was used as a low iron minimal medium (37). Glassware for Tris medium was treated as described previously (37). For iron-replete conditions, Tris medium was supplemented with ferric chrysobactin at the concentrations indicated. Ferric chrysobactin was prepared by adding FeCl3 to chrysobactin in 0.1 mM Tris, pH 7.5, at a ligand/iron ratio of 3:1. Glucose (2 g/liter) was used as carbon source. The antibacterial agents and chemicals were used as reported previously (35, 37) unless otherwise specified.

Construction of the dps and bfd Deficient Mutants and General DNA Methods—A genomic fragment from the dps locus was amplified by PCR with the primers 5′- sense dps1s (5′-attatgcttgctggga-3′) and 3′- reverse dps1r (5′-catcaaaagctcttcct-3′) and cloned using the pGEM-T Easy vector. This fragment was then subcloned into the pBC plasmid using appropriate restriction enzymes to introduce a unique EcoRV restriction site into the dps gene. The Ω-Spec interposon from pHP45Ω hydrolyzed with SmaI was inserted into the EcoRV site to obtain plasmid pAB12 (38, 39). To introduce a nonpolar mutation in the bfd gene, the alpha-3 cassette, obtained by SmaI digestion of pUC18K, was inserted into the T4 Polymerase blunted NsiI site of pAB3 (40). Plasmid transformation and marker exchange recombination with the chromosome were performed as described previously (37). Correct recombination was confirmed by PCR and Southern blot hybridization experiments. For construction of plasmid pAB14, a genomic fragment from the bfr-bfd encoding locus was amplified by PCR with the primers sense bfr1s (5′-ggtcgtagagccgga-3′) and reverse bfr1r (5′-gtagaggcttcacacag-3′) and inserted into the pGEM-T Easy vector. This fragment was subcloned into the Apal and SpeI sites of the pBC plasmid. DNA manipulation techniques (chromosomal DNA isolation, cloning, and electrophoresis) were described previously (35). Plasmids were extracted using the QIAprep spin miniprep kit (Qiagen). All of the cloning experiments were performed in the DH5α strain of E. coli. PCR was performed in a DNA thermocycler (Hybaid PCR Express System) with denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, an extension at 72 °C for 1 min. PCR products were cloned using the plasmid pGEM-T Easy, according to the manufacturer’s instructions. Nucleotide sequences of PCR products and plasmids were obtained from Genome Express.

In Vivo Labeling of Bacterial Cultures, Preparation of Whole Cell Extracts, and Analysis of Protein-bound 59Fe Iron—An overnight culture in L broth of the strain to be studied was...
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dilated 1:40 in 5 ml of Tris medium supplemented with glucose
placed in a 50-ml Erlenmeyer flask and incubated with shaking
until $A_{600\text{ nm}} = 0.4$ was reached. Iron labeling was started by
adding a ferric chrysobactin complex solution prepared with
$^{59}\text{FeCl}_3$ (3–20 mCi/mg iron in 0.5 m HCl; GE Healthcare). Sam-
ples (1 ml) were taken at the indicated times. Excess unlabeled
ferric chrysobactin was added to each sample. Bacterial cells
were spun down in a microcentrifuge, at 7000 RPM at 4 °C. The
cells were washed in a solution containing 50 m potassium
phosphate, pH 7.8, 0.1 mM EDTA, and 10 mM MgCl$_2$ and har-
vested by centrifugation. The bacterial pellets were resus-
pended in 20–40 $\mu$l of the same solution with DNase I and
lysozyme added at final concentrations of 0.1 and 0.2 mg/ml,
respectively, and incubated for 30 min at 4 °C. The cells were
lysed by six freeze/thaw cycles. The extracts were centrifuged
at 15,000 × g for 5 min, and supernatant fluids were kept at
−20 °C. To analyze the fate of iron during bacterial growth,
labeled cells were centrifuged at 3000 × g at 4 °C for 15 min.
The cells were washed twice in Tris medium; washing fluids
were eliminated by centrifugation. The washed cells were resus-
pended in Tris medium with glucose and left to grow for 2 h. The samples (1 ml) were taken at the indicated times, and
bacterial cells were treated as described above. Whole cell
extracts (25 $\mu$g protein) were analyzed by native PAGE in 10%
acrylamide and Tris-glycine buffer. Protein concentration was
determined by the Bradford method (41). The dried gels were
autoradiographed at −80 °C, for 48 h, using KODAK BioMax
XAR films. For each bacterial strain, six independent time
course experiments were performed. The autoradiograms
shown are representative of one experiment. For each time
point, the amount of $^{59}$Fe bound to relevant proteins was deter-
mined by scintillation counting of the excised bands from the
gels. The results are given as the relative percentages of total
counts measured on the gels, for each lane. The data reported
are the averages of six independent experiments, and the stand-
ard deviations are indicated.

**Immunodetection of the Bfr Protein**—Proteins (25 $\mu$g) were
loaded and run onto 15% polyacrylamide 0.1% SDS denaturat-
ing gels. The proteins were transferred onto nitrocellulose
membranes (Protran BA 83; Whatman) at 350 mA for 75 min in
30 m Tris, 192 m glycine, 0.025% SDS, 20% v/v methanol, pH
8.3, using the Bio-Rad mini-Trans-Blot electrophoretic transfer
cell. Bfr antiserum was used at a dilution of 1/4000. Antibody
binding was detected with goat anti-rabbit immunoglobulin
conjugated to alkaline phosphatase.

**Analysis of FMP Iron and Mass Spectrometry**—Bands corre-
sponding to the $^{59}$Fe-labeled proteins doublet detected by auto-
radiography of native gels were cut out (Fig. 5A, panel 1) and
analyzed by SDS-PAGE in 10% acrylamide and Tris-glycine
buffer (Fig. 5A, panel 3), as described by Schägger and Von
Jagow (42). The gels were stained with Coomassie Blue. For
control experiments, the protein bands excised from the gel
were analyzed a second time on native PAGE (Fig. 5A, panel 2)
to check for the presence of a $^{59}$Fe spot corresponding to the
protein doublet detected on the first gel. Individual spots visu-
alized after SDS-PAGE were excised, reduced with dithiothe-
ritol, alkylated with iodoacetamide, and digested with trypsin
overnight at 37 °C. Tryptinized peptides were extracted from
the gel pieces with 50% acetonitrile, 0.1% trifluoroacetic acid,
concentrated, desalted, using a ZipTip (Millipore, Molsheim,
France), and spotted onto a steel target with $\alpha$-cyano-4-hy-
droxycinnamic acid as a matrix. The peptide mass fingerprint
was acquired after external calibration with ions from des-
Arg$^\text{1}$-Bradykinin, Angiotensin I, Glu$^\text{1}$-Fibrinopeptide B, and
neurotensin, on a 4800 TOF-TOF spectrometer (Applied Bio-
systems) equipped with a YAG-200 Hz laser (355 nm). Operat-
ing parameters for the Reflectron included 1500 laser shots/
spectrum. Monoisotopic masses were used with a maximum
deviation of ±100 ppm for mass assignment. For protein iden-
tification, the peptide mass fingerprint was searched against the
ASAP data base, using Mascot (Matrix Science, London, UK).
Protein hits were accepted if the Mascot score was greater than
the significance threshold. This procedure was carried out in six
independent samples isolated from cytosolic extracts of paren-
tal cells supplied with $^{59}$Fe-chrysobactin at concentrations of
0.25 $\mu$M, for 40 min.

**Transport Assays**—Bacterial cultures were grown in the same
conditions used for in vivo labeling experiments. Transport
assays were carried out as described by Rauscher et al. (43), with
the addition of $^{59}$Fe-labeled chrysobactin to the transport
medium at a concentration of 0.25 $\mu$M.

**Enzyme Assays**—Aconitase activity was assayed in bacterial
pellets from 100-ml cultures grown in the conditions indi-
cated and stored at −80 °C for 24 h, as described by Gardner
(44). The protein concentration was determined by the
Bradford method.

**RESULTS**

**Intracellular Distribution of Iron in Cells Supplied with
$^{59}$Fe-Chrysobactin**—Previous studies have shown that the ferric
chrysobactin complex is dissociated inside the cell through a
rapid reductive process, making iron available for metabolic
needs (43). Thus, we used $^{59}$Fe-chrysobactin to try to identify
intracellular protein targets of iron in E. chrysanthemi cells. We
used a double mutant strain deficient in biosynthesis of both
chrysobactin and achromobactin (PPV20) to avoid iron exchange
between both ligands. In the low iron minimal Tris
medium, the doubling time of this mutant was 160 min. The
addition of ferric chrysobactin to the medium stimulated the
growth rate, the doubling time being 100 min. Cytosolic protein
extracts were analyzed by PAGE on nondenaturing gels, and
protein-bound iron was visualized by autoradiography as des-
cribed under “Experimental Procedures.” We first tested a
concentration range of 0.06–1 $\mu$M $^{59}$Fe-chrysobactin added
over a period of 40 min (Fig. 1A). We detected a continuum of
bands increasing in intensity with higher $^{59}$Fe concentrations,
which could correspond to the banding region revealed by Coo-
massie Blue staining. In addition, two strong $^{59}$Fe signals (des-
ignated as I and 2 on Fig. 1A), a slowly migrating protein species
and a quickly migrating protein doublet, were apparent. A third
signal (designated as 3 on Fig. 1A) became visible with concen-
trations of $^{59}$Fe higher than 0.125 $\mu$M. We then performed a
time course experiment. Protein-bound iron was probed at
10-min intervals over a 40-min period after adding 0.25 $\mu$M
$^{59}$Fe-chrysobactin (Fig. 1B). We observed the same $^{59}$Fe signals
increasing in intensity with time. Thus, iron binds a wide vari-
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The doublet corresponding to the quickly migrating protein species was present in all of the mutant strains. Its signal intensity increased over time, as observed for the parental strain (supplemental Fig. S1). Thus, some of the iron released from chrysobactin is sequestered by Bfr, Dps, and a set of proteins unrelated to ferritins, designated FMP. The amounts of $^{59}$Fe bound to these various protein species were quantified (supplemental Table S2). In parental cells supplied with 0.25 μM $^{59}$Fe-chrysobactin for 40 min, iron bound to Bfr represented ~5%, Dps iron represented ~1%, and FMP iron 16% of total $^{59}$Fe detected on the gels. In the bfr mutant, levels of FMP iron were similar to those of the parent strain, whereas they reached 20% in the dps mutant. In the fur mutant, iron bound to Dps and FMP represented 16 and 20% of total $^{59}$Fe signal, respectively. We did not detect iron bound to FtnA, probably because the ftnA gene is not expressed in iron-deprived cells (32).

Release of Iron from Bacterioferritin Requires Bacterioferredoxin—The roles of bacterioferritin and bacterioferredoxin in iron metabolism are not well understood. As in E. coli, E. chrysanthemi bacterioferritin is not involved in long term iron storage (21, 32). To gain insight into the roles of these proteins, we constructed a nonpolar bfd-negative mutant (PPV55) and examined the distribution of iron in this mutant, and in the parental strain, over time. Cultures of iron-depleted cells were supplied with 0.25 μM $^{59}$Fe-chrysobactin for 40 min (Fig. 2A). In the bfr mutant, the signal intensity for Bfr-bound iron was 2.8 times greater than in the parental strain. However, the intensity of signals corresponding to Dps and FMP, was similar in both strains (supplemental Table S2). We carried out the same

FIGURE 1. Native PAGE analysis of protein extracts from E. chrysanthemi cells supplied with $^{59}$Fe-chrysobactin. Protein-bound iron was detected by autoradiography. Further details are described under “Experimental Procedures.” A, $^{59}$Fe-chrysobactin was supplied to parental cells at the indicated concentrations for 40 min. The autoradiogram shown in the right panel corresponds to proteins visualized by Coomassie Blue staining in the left panel. B, 0.25 μM $^{59}$Fe-chrysobactin was supplied to parental cells for the indicated times. C, parental and relevant mutant cells (as indicated by P and corresponding genotypes) were supplied with 1 μM $^{59}$Fe-chrysobactin for 40 min. Insoluble material is visible at the tops of some lanes. $^{59}$Fe-labeled signals are designated by arrows 1, 2, and 3, respectively.

FIGURE 2. Distribution of iron in bacterioferredoxin-proficient and -deficient cells supplied with $^{59}$Fe-chrysobactin. Native PAGE analysis of protein extracts was performed as described in Fig. 1. A, $^{59}$Fe-chrysobactin was supplied at the concentration of 0.25 μM for the indicated times. B, $^{59}$Fe-chrysobactin was supplied at the concentration of 0.5 μM for 40 min as indicated. The cells were then washed and grown for the indicated times (details are under “Experimental Procedures”); optical density at 600 nm (OD$_{600}$) of bacterial cultures is indicated. The strains are designated as in Fig. 1. Plasmid pAB14 contains the bfd gene under the control of its own promoter.
experiment with a bacterial construct containing extra copies of the bfd gene expressed on a multicopy plasmid (pAB14) (Fig. 2A). The signal intensity for Bfr iron was strongly reduced, whereas that observed for Dps was increased. We then analyzed the fate of iron at different stages of cell growth. Iron-depleted cultures from the parental strain and the bfd mutant were treated with 0.5 μM 59Fe-chrysobactin for 40 min. The cells were washed and grown in fresh iron-free medium for an additional 2 h (see “Experimental Procedures”). In parental cells, the signal intensity for Bfr iron progressively decreased, indicative of the metal being released during cell growth. We did not observe this effect in bfd cells. This suggests that bacterioferredoxin is required for iron release from bacterioferritin. The absence of Bfr-bound iron from cells overexpressing the bfd gene is likely to be due to overproduction of the Bfd protein causing an exacerbation of the release process. These data highlight the importance of a clustered organization of the bfd and bfr genes in an operon, allowing the coordinated production of Bfd and Bfr proteins.

Delivery of Iron to Bacterioferritin Is Impaired in Nonpolar sufA, sufB, sufD, sufS, and sufE Mutants—The sufABCDSE operon is involved in iron-sulfur cluster biogenesis and is iron-regulated (13). We first compared the growth of nonpolar sufA to sufE negative mutants (PPV56–PPV61) and bfr and bfd mutants (PPV51 and PPV55) in Tris medium, with or without ferric chrysobactin (Fig. 3A). In Tris medium, all of the strains grew slowly, although to various extents depending on the genotype. Cell growth was most severely affected in the sufA, sufC, sufS, and bfd mutants (p value ≤ 0.03). The addition of ferric chrysobactin greatly stimulated growth of the parental strain and to a lesser extent that of the mutants. Growth of the sufC mutant was only mildly stimulated. To confirm that these growth defects were not caused by impaired ferric chrysobactin.
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We then analyzed the distribution of iron in the suf negative mutants (PPV56–PPV61) over time. Iron-depleted cultures from the mutants were treated with 0.25 μM 59Fe-chrysobactin for 40 min (Fig. 3B). Parental and bfr- and bfd-deficient cells contained similar amounts of total 59Fe. In the sufA, sufD, sufS, sufE, and fur mutants, 59Fe levels were 40% higher than those in the parental strain (p value ≤ 0.04). sufS and sufE mutants had similar levels to those observed in the fur mutant, in which iron transport is derepressed.

We then analyzed the distribution of iron in the suf negative mutants (PPV56–PPV61) over time. Iron-depleted cultures from the mutants were treated with 0.25 μM 59Fe-chrysobactin for 40 min, and protein iron was analyzed (Fig. 4A). We did not detect a signal corresponding to Bfr-bound iron in sufA and sufB mutants, and the signal in the sufD, sufS, and sufE mutants was very weak. The amount of Dps-bound iron was higher in these mutants than in the parental strain (Fig. 4A and supplemental Table S2). In the sufC mutant, iron sequestered by Bfr seemed to accumulate over time; the amount of Bfr iron measured at 30 min was twice as high as that observed for the parental strain. The amount of Dps iron in this mutant was similar to that of the parental strain. FMP iron was detected in all suf mutants. The amount of FMP iron was higher in the sufC, sufS, and sufE mutants than in the parental strain. We checked by immunoblotting that the lack of Bfr iron in the suf mutants was not due to the absence of Bfr protein (Fig. 4B). Given that the sufC mutant was able to accumulate more iron in bacterioferritin than the parental strain, we examined the turnover of this metal during cell growth, as described for the bfd mutant above. We did not observe the release of iron from bacterioferritin during cell growth (Fig. 4C). Thus, the sufC and bfd mutations result in a similar phenotype involving a defect in iron release from bacterioferritin.

Aconitase Activity Is Impaired in Nonpolar sufA, sufB, sufD, sufS, and sufE Mutants—To determine whether iron recycled from bacterioferritin is essential to the formation of iron-sulfur clusters through the Suf pathway, we measured aconitase activity in parental and mutant strains. E. chrysanthemi contains an ortholog of the E. coli aconitase B-encoding gene acnB. Aconitase B is a member of the iron-sulfur-containing protein family (43). We determined the activity of this enzyme in cell extracts of iron-depleted cultures treated with or without ferric chrysobactin in the parental strain (Fig. 4D). Ferric chrysobactin-treated cultures displayed significant levels of aconitase activity, whereas aconitase activity was 10 times lower in cells exposed to the conditions of iron depletion.

Characterization of FMP Iron Pool—The 59Fe-labeled doublet detected on native gel was further resolved to reveal its individual polypeptides by denaturing SDS-PAGE as described under “Experimental Procedures” and in Fig. 5A. We identified several spots, ranging from 3 to 10, depending on the experiment considered. The corresponding polypeptides were analyzed by mass spectrometry. These polypeptides were related to basic biological processes, such as protein translation and folding, energy/carbon metabolism, amino acid biosynthesis, and potential metal ion binding (supplemental Table S3). FMP iron may thus play a role in protein translation. Given that magnesium ions contribute to maintenance of the 50 S ribosomal architecture and is necessary for EF-Tu protein chain elongation activity (46, 47), we checked that ferrous ions were not replacing magnesium ions. Increasing the magnesium concentration in the culture medium up to 20 mM, i.e. 0.5 mM in Tris medium, did not affect formation of this pool (Fig. 5B). We analyzed the effect of antibiotics - fusidic acid, which blocks
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FIGURE 5. FMP iron analysis. A, FMP iron was analyzed as described under “Experimental Procedures.” Panel 1, excision of the gel band corresponding to signal 2 in Fig. 1B; Panel 2, second migration on native gel: autoradiogram (left side) and Coomassie Blue staining (right side); Panel 3, individual spots revealed by Coomassie Blue staining after SDS-PAGE (left lane); from top to bottom, EF-G factor, aconitase, 30S ribosomal protein S1, HSP-90, trigger factor, argininosuccinate synthetase, serine hydroxymethyltransferase, aspartate-semialdehyde dehydrogenase. The apparent molecular sizes of standard proteins (right lane) in kDa are indicated. B, distribution of iron in parental cells exposed to agents inhibiting protein translation. Native PAGE analysis of protein extracts was performed as described in Fig. 1. The cells were grown in Tris medium containing the concentrations of MgCl2 indicated, and 59Fe-chrysobactin was supplied at the concentration of 0.125 μM. Nalidixic acid, fusidic acid, and tetracycline were added to bacterial cultures at a concentration of 10 μg, 1.2 mg, and 2.5 μg/ml respectively, 5 min before the addition of 0.5 μM 59Fe-chrysobactin.

EF-G–mediated translocation of peptidyl-tRNA on the ribosome, and tetracycline, which disrupts codon/anticodon interactions - on this iron pool (48, 49) (Fig. 5B). These drugs were added to cultures 5 min before adding 59Fe-chrysobactin. Formation of the pool of FMP iron was severely disrupted. The continuum of bands was also strongly reduced, whereas the pool of iron bound to bacterioferritin remained unchanged. Nalidixic acid, an inhibitor of DNA gyrase (50), had no effect. Thus, de novo protein biosynthesis is required for detection of 59Fe signals corresponding to FMP and to the continuum of protein bands.

DISCUSSION

In this study, we investigated the intracellular fate of iron transported by a siderophore-mediated pathway. We provide a model demonstrating the management of iron in bacterial cells during a period of transition, in this case from conditions of iron depletion to iron repletion. We identified a functional link between the Suf machinery and the bacterioferritin-bacterioferredoxin system, which results in a balanced distribution of iron to its protein targets and optimization of the metabolic role of this metal.

We used PAGE under non-denaturing conditions to detect 59Fe-labeled proteins in soluble extracts from E. chrysanthemi cells supplied with 59Fe via chrysobactin. We observed a diverse range 59Fe signals differing in intensity, visualized as a continuum of faint bands and intense bands corresponding to FMP iron and iron bound to bacterioferritin and mini-ferritin. At 40 min after the addition of the iron source, ~78% of total 59Fe counts measured on the gels could be attributed to this continuum, ~16% could be attributed to FMP, 5% to bacterioferritin, and 1% to mini-ferritin. The amounts of iron associated to the continuum and to FMP were strongly reduced by inhibiting protein translation with antibiotics. These data suggest that incoming iron is delivered to a wide range of protein species visualized as a continuum, which probably includes many metalloenzymes and other metalloproteins. This process requires de novo protein biosynthesis; thus, the delivery of iron to these targets seems to be mediated by newly synthesized intracellular metal carrier(s). It is also possible that the whole continuum of bands corresponds to a pool of newly translated proteins having acquired their iron cofactor. This possibility fits with the presence of the FMP iron pool, also resulting from de novo protein biosynthesis. However, we were surprised not to find that iron-binding proteins could account for the high metal content observed in this pool. The isolated proteins were involved in basic cellular processes such as protein translation and folding, carbon metabolism, amino acid biosynthesis, and possibly metal ion binding. They must be the most abundant species in cells recovering active metabolism. Iron-starved cells are metabolically weak. The addition of 59Fe-chrysobactin rapidly stimulated growth in the parental strain, indicative of metabolic rescue (Fig. 3). Consistent with this, signal corresponding to FMP iron was weak when bacterial cells were grown in rich medium supplemented with 59Fe (data not shown). This suggests that the strong 59Fe signal observed for the FMP pool results from an accumulation of iron because of a set of iron-binding proteins migrating together on native gels. The amount of each protein species would be too low to be detectable. An alternative but not exclusive possibility would be the presence of low levels of an unknown protein able to accommodate high levels of iron. Further investigations are required to address these issues.

Bacterioferritin and to a lesser extent mini-ferritin also appear as primary targets of intracellular iron delivery, indicating that these iron storage proteins are important in the metabolism of iron-starved cells. In the absence of bacterioferritin, as in the bfr mutant, mini-ferritin can store more iron. This effect is particularly marked in a fur mutant that has increased levels of Dps-bound iron, probably because of the constitutive expression of its iron transport proteins (Fig. 3B), reduced transcription of the bfr gene (32), and increased transcription of the dps gene.4 The role played by the bacterioferritin is interesting. Indeed, we found that iron bound to this protein was absent from sufA, sufB, and sufD mutants and strongly reduced in mutants harboring a sufS or sufE mutation. This defect was not due to the absence of Bfr protein, as shown by immunoblotting. By contrast, the sufC mutant displays increased levels of Bfr iron. This suggests that the Suf components, with the exception of SufC, are involved in the transfer of iron to bacterioferritin. Although the importance of Suf machinery in iron-sulfur cluster assembly under conditions of iron limitation is established, the mechanisms underlying Suf protein function in vivo are poorly understood. In the model proposed by Layer et al. (51), SufS is a cysteine desulfurase that releases sulfur from cysteine

4 D. Expert, A. Boughammoura, and T. Franzia, unpublished observations.
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and SufE is a sulfur transfer protein donating sulfur to SufB and SufA (Fig. 6). SufB and SufA can act as scaffolding proteins and mediate transfer of clusters to target apoproteins. SufC is an ATPase subunit of the ABC transporter family able to form a complex with both SufB and SufD. SufB can also interact with SufE if SufC is present. It remains to be determined how iron is handled by this machinery.

Thus, we can propose a model involving the release of iron from the siderophore, its transfer to the Suf pathway, possibly via SufA, SufB, and/or SufD, followed by its incorporation by bacterioferritin. Bfr iron seems to be recycled through the action of bacterioferredoxin, because iron release from Bfr was compromised in a bfd mutant. This is consistent with previous data showing that the E. coli Bfd protein can physically interact with Bfr and acts as a redox catalyst (34, 52). However, iron recycled through the Bfr-Bfd pathway does not seem to be essential for the function of Suf machinery, given that the bfd mutation had no effect on the production of aconitase. The role of the SufC component could be to facilitate the formation of iron-sulfur clusters and/or their transfer to apotargets, a function that is reminiscent of that of the ATPase ApbC from S. enterica (53). Indeed, aconitase activity in the sufC mutant is only reduced by a factor of two, demonstrating that the Suf machinery, although less efficient, is still functional. This mutant, similarly to the bfd mutant, has defective iron recycling from bacterioferritin. Thus, the production of bacterioferredoxin may be impaired in the sufC mutant, a defect that could be due to the reduced capacity of this strain to produce or transfer iron-sulfur clusters. The validity of this model implies that Suf, Bfr, and Bfd proteins cooperate in various configurations in vivo, although other iron trafficking proteins may be involved. In E. coli, the protein NfuA could act as a scaffold/chaperone for the insertion of iron-sulfur clusters into target proteins (54). Thus, the role of the E. chrysanthemi NfuA protein has to be elucidated. Other findings suggest that the YggX small protein identified in Salmonella enterica for its protective role against oxidative stress facilitates iron trafficking to appropriate cellular locations (55–57). The E. chrysanthemi genome encodes an YggX homolog. Further study is needed to determine the potential role of this protein. Nevertheless, the YggX protein binds ferrous ions only weakly, and thus a role in iron trafficking is unlikely (58). Another possible candidate is the homolog of Ytfe, a di-iron protein conserved in enterobacteria and present in E. coli under conditions of nitrosative stress and iron starvation (59, 60).

All of the mutations studied impairing the Bfr-Bfd pathway or the Suf components reduced cell growth even under conditions of iron supplementation with chrysoerbactin. These mutations have no effect on ferric chrysoerbactin transport per se; therefore, the observed phenotypes result from impaired intracellular iron management. Indeed, the sufA, sufB, sufD, sufS, and sufE mutants have a deficit in iron-sulfur clusters and cannot store iron through sequestration by bacterioferritin, even after 3 h of iron supply (data not shown). Growth of the sufC mutant was severely affected, although it still produces significant levels of aconitase. In fact, this mutant is particularly sensitive to oxidative conditions, including agents like streptonigrin (13). However, unlike the sufA, sufD, sufS, and sufE mutants, it did not accumulate more iron than normal (Fig. 3B). This suggests that the reduced growth of this mutant could be due to the activity of the other Suf proteins causing accumulation of incompletely processed iron-sulfur clusters prone to oxidative damage (3). Cell growth was also significantly impaired in the bfd mutant to a greater extent than that observed for the bfr mutant. Thus, the Bfr-Bfd pathway seems to be needed for a rapid turnover and optimized intracellular utilization of iron.

In conclusion, the selective incorporation of iron into iron-sulfur cluster proteins and other proteins requiring this metal as a cofactor involves the action of Suf components, Bfr and Bfd proteins. This can be beneficial to many pathogens for growth in a host environment that is continually changing in terms of iron availability and redox conditions.

Acknowledgments—We thank J.-M. Camadro and J.-J. Montague for access to the Plateforme Protéomique (Institut Jacques Monod, CNRS-Université Paris 6 and 7) and helpful discussions. We thank S. Andrews for the gift of E. coli bacterioferritin antisera. We are grateful to B. Matzanke for its interest in this work. We are grateful to J. Gray for English reading of the manuscript.

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