Chrysobactin-dependent Iron Acquisition in *Erwinia chrysanthemi*

**FUNCTIONAL STUDY OF A HOMOLOG OF THE *ESCHERICHIA COLI* FERRIC ENTEROBACTIN ESTERASE**

Received for publication, August 7, 2001, and in revised form, October 19, 2001

Published, JBC Papers in Press, November 1, 2001, DOI 10.1074/jbc.M107530200

Lise Rauscher‡, Dominique Expert‡‡, Berthold F. Matzanke¶, and Alfred X. Trautwein**

From the ‡Laboratoire de Pathologie Végétale, UMR 217 Institut National de la Recherche Agronomique, Institut National Agronomique Paris-Grignon, Université Paris 6, 16 rue Claude Bernard, 75231 Paris Cedex 05, France and the ¶Medical University Lübeck, the **Institute of Physics and the ‡‡Isotope Laboratory, Ratzeburger Alle 160, D-23538 Lübeck, Germany

Under iron limitation, the plant pathogen *Erwinia chrysanthemi* produces the catechol-type siderophore chrysobactin, which acts as a virulence factor. It can also use enterobactin as a xenosiderophore. We began this work by sequencing the 5′-upstream region of the fet-cbsCEBA operon, which encodes the ferric chrysobactin receptor and proteins involved in synthesis of the catechol moiety. We identified a new iron-regulated gene (*cbsH*) transcribed divergently relative to the fet gene, the translated sequence of which is 45.6% identical to that of *Escherichia coli* ferric enterobactin esterase. Insertions within this gene interrupt the chrysobactin biosynthetic pathway by exerting a polar effect on a downstream gene with some sequence identity to the *fct-cbsCEBA* operon, which encodes the ferric chry-sobactin receptor and proteins involved in synthesis of the catechol moiety. We identified a new iron-regulated gene (*cbsH*) transcribed divergently relative to the fet gene, the translated sequence of which is 45.6% identical to that of *Escherichia coli* ferric enterobactin esterase. Insertions within this gene interrupt the chrysobactin biosynthetic pathway by exerting a polar effect on a downstream gene with some sequence identity to the *E. coli* enterobactin synthase gene. These mutations had no effect on the ability of the bacterium to obtain iron from enterobactin, showing that a functional *cbsH* gene is not required for iron removal from ferric enterobactin in *E. chrysanthemi*. The *cbsH*-negative mutants were less able to utilize ferric chrysobactin, and this effect was not caused by a defect in transport *per se*. In a nonpolar *cbsH*-negative mutant, chrysobactin accumulated intracellularly. These defects were rescued by the *cbsH* gene supplied on a plasmid. The amino acid sequence of the CbsH protein revealed characteristics of the S9 prolyl oligopeptidase family. Ferric chrysobactin hydrolysis was detected in cell extracts from a *cbsH*-positive strain that was inhibited by diisopropyl fluoro-phosphate. These data are consistent with the fact that chrysobactin is a D-lysyl-L-serine derivative. Mössbauer spectroscopy of whole cells at various states of 57Fe-labeled chrysobactin uptake showed that this enzyme is not required for iron removal from chrysobactin in vivo.

The CbsH protein may therefore be regarded as a peptidase that prevents the bacterial cells from being intracellularly iron-depleted by chrysobactin.

Iron is an essential but nevertheless potentially toxic element for most living organisms. The bioavailability of the ferric ion is extremely limited because of its poor solubility (at pH 7, $K_{sp} = 10^{-17}$ M). A wide variety of microorganisms accommodate this situation by excreting siderophores. Siderophores are high-affinity Fe(III)-scavenging/solubilizing molecules that, once loaded with iron, are specifically imported into the cell. In *Escherichia coli* K12, it has been demonstrated that delivery of the ferric siderophore complex to the cell implicates active transport (1, 2). The passage through the outer membrane requires a receptor that is a pore energized by cytoplasmic membrane-generated proton-motive force transduced by the TonB protein. The ferric complex then binds to the periplasmic component of a permease belonging to the ABC transporter family, which completes the passage to the cytosol. The fate of the ferric siderophore complex in the cytosol is not clearly understood. As the stability constants of ferric siderophore complexes are very high and the ferrous complexes dissociate near neutral pH, enzymatic reduction to the ferrous state has been proposed to be a plausible mechanism for iron removal (3, 4). Ferric siderophore reductase activity has been found in cell extracts from several microorganisms (5–8). However, the redox potentials for hexadentate catechol siderophores are out of the range of physiological reductants, and it is assumed that ligand degradation is required for transformation of the irreducible form of the complex into a reducible one. In *E. coli*, the ester bonds of the siderophore enterobactin (enterochelin), the cyclic trimer of 2,3-dihydroxybenzoyl-L-serine (9) (see Fig. 1), are hydrolyzed by the ferric enterobactin esterase encoded by the *fes* gene, yielding 2,3-dihydroxybenzoyl-L-serine (10–12). The redox potential of this compound is 2 orders of magnitude below that of ferric enterobactin. *fes*-negative mutants fail to grow if ferric enterobactin is the only iron source (13, 14). The plant pathogenic enterobacterium *Erwinia chrysanthemi* strain 3937 provides another illustration of this problem.

Under iron limitation, *E. chrysanthemi* produces the catechol siderophore chrysobactin. This siderophore is essential for this pathogen to disseminate throughout its host plant and to cause systemic soft-rot symptoms (15). Chrysobactin is a bidentate ligand consisting of a monomer of 2,3-dihydroxybenzoyl-L-lysyl-L-serine (16) (see Fig. 1). Ferric chrysobactin is transported into the cell via its specific TonB-dependent outer membrane receptor Fct (17–19) and a cytoplasmic membrane permease that is missing in a class of mutants deficient in ferric chrysobactin uptake (20). These mutants do not acquire iron from ferric enterobactin. Enterobactin is not synthesized by *E. chrysanthemi* cells, but promotes growth of a chrysobactin-deficient mutant if supplied exogenously. An iron-regulated outer membrane protein with an apparent molecular mass of 88,000 Da and immunologically related to the *E. coli* ferric enterobactin receptor FepA is thought to play a similar function in *E. chrysanthemi* (21). In addition, *E. chrysanthemi*

*This work was supported by grants from the Institut National de la Recherche Agronomique. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank**EBI Data Bank with accession number(s) AF011334.

§ Researcher from CNRS. To whom correspondence should be addressed. Tel.: 33-1-44-08-17-06; Fax: 33-1-44-08-16-31; E-mail: expert@inapg.inra.fr.

This paper is available on line at http://www.jbc.org
strain 3937 produces another high-affinity iron uptake system mediated by a citrate siderophore called achromobactin (22).

Most of the proteins involved in chrysobactin-mediated iron transport are encoded by a 50-kb contiguous region of the *E. chrysanthemi* chromosome (20). The fct-cbsCEBA operon codes for the receptor Fct and the enzymes leading to the catechol moiety in chrysobactin biosynthesis (23) (see Fig. 1). Analysis of the fct gene sequence (18) revealed a strong resemblance of the promoter region to the bidirectional promoter controlling *EntF* proteins, respectively, which are two components of the *fur* sequences (Fur or iron boxes) (33, 34).

**FIG. 1.** Enterobactin- and chrysobactin-mediated iron transport in *E. coli* K12 (70) and *E. chrysanthemi* strain 3937, respectively. Upper, structures of enterobactin and chrysobactin are also shown. Enzymatic cleavage sites are indicated by arrows. Lower, gene clusters specifically involved in transport and biosynthetic pathways are shown. Details are given in the Introduction. Arrows indicate the direction of transcription. Filled arrows correspond to the genes referred to in the Introduction.

Bacterial Strains, Plasmids, and Microbiological Techniques—The abbreviations used are: EDDHA, ethylenediamine-\(\text{H}_2\)/\(\text{H}\); N,N,N,N-bis(2-hydroxyphenylacetic acid); ORF, open reading frame.

DNA templates were isolated from cultures of *E. chrysanthemi* strain 3937 and *E. coli* strain JM101 harboring pCS1 and grown in Tris medium, reaching an absorbance at 600 nm of 0.8 and 0.6 for high- and low-iron conditions, respectively. DNA isolation and primer extension analyses were performed as described previously (18). A 32P-labeled oligonucleotide complementary to nucleotides 158–142 was used for primer extension.

**Identification of the CbsH Product**—The procedure described by Tabor and Richardson (42) was used to produce proteins encoded by the pT7 derivative plasmid. Samples were radiolabeled as described previously (37). Proteins were separated by electrophoresis on SDS-8% polyacrylamide gel.

**Siderophore Detection**—Catechol was determined by the chemical assay of Arnow (43) using dihydroxybenzoic acid as the standard. Siderophore activity was detected as chrome azurol S-reacting material in the culture supernatant (44) using desferrioxamine B (Desferal, Novartis Pharma S. A., Rueil Malmaison, France) as the standard. The biological activities of chrysobactin and enterobactin were determined in bioassays as described previously (16) using strains BW193 and RW818-60 for enterobactin and strains L2 *cbsE-I* and L2 *fct-34* for chrysobactin as indicators.

**Quantitative Determination of Ferric Chrysobactin in Cell Lysates**—A culture (grown exponentially in L broth) of the strain to be studied was diluted 1:40 in 20 ml of Tris medium supplemented with glucose and 5 \(\mu\)M FeCl₃. Cultures were grown aerobically for 12–14 h. Cells were washed, suspended in 1 ml of Tris medium, and disrupted in a Vibra Cell apparatus (Sonic and Materials Inc.). The lysis mixture was centrifuged at 7000 rpm for 30 min at 4°C in a microcentrifuge (Medical Scientific Equipment, Leicester, United Kingdom). Pelleted cell debris was discarded, and the supernatant was checked for the presence of ferric chrysobactin in a bioassay as described previously (16) using strains BW193 and RW818-60 for enterobactin and strains L2 *cbsE-I* and L2 *fct-34* for chrysobactin as indicators.

**Assay for Ferric Enterobactin Esterase Activity**—Hydrolysis of ferric chrysobactin was assayed in cell extracts from the bacterial strains tested and prepared as follow. Cells were grown aerobically in 10 ml of Tris medium supplemented with glucose until an absorbance at 600 nm of 0.6–0.9 was reached. Cells were washed, suspended in 2 ml of 0.1 M Tris- HCl (pH 7.5) and disrupted as described above. Lysates were supplemented with dithiothreitol at a final concentration of 0.005 mM and centrifuged for 20 min at 20,000 x g. The enzymatic activity in the supernatants was immediately tested. The reaction mixture (incubated at 37°C for 1 h) contained 0.300 ml of lysozyme, 0.430 ml of 0.1 M Tris- HCl (pH 7.5), and 0.020 ml of the bis complex of ferric chrysobactin to give a final concentration of 0.028 mM. Ferric chrysobactin was prepared by adding FeCl₃ to chrysobactin in 0.1 M Tris (pH 7.5) at a ligand/iron ratio of 4:1. Chrysobactin (a gift from Dr. J. S. Buyer) was synthesized according to the procedure described previously (46). Protein concentration in cell lysates was determined with the Bradford reagent. Hydrolytic activity was determined spectrophotometrically as described above. Enzymatic activity is expressed in nanomoles of ferric chrysobactin hydrolyzed per mg of protein/h. Disopropyl fluorophosphate was added to the reaction mixture at a final concentration of 0.036 mM. For each strain, three independent experiments were performed.

**Vicia faba Ferric Enterobactin Esterase Activity**—For ferric enterobactin esterase activity, cell extracts from the bacterial strains tested were prepared as described above. Lysates were assayed as reported by Langman et al. (13). Enzymatic activity is expressed in nanomoles of enterobactin hydrolyzed per mg of protein/h. For each strain, three independent experiments were performed. Ferric enterobactin was prepared according to the procedure reported by Greenwood and Luke (10), with modifications. The supernatant of a 10-liter culture of *E. coli* strain
BZB1013 was lyophilized and extracted with ethyl acetate. As a final purification step, ferric enterobactin dissolved in methanol was passed through a column of Sephadex LH-20 (30 g) with methanol as the eluent. Fractions were collected, evaporated, and dissolved in 0.1 M phosphate buffer (pH 7). Catechol-positive fractions were bioassayed using indicators RW193 and RW158-60 as indicators and checked by ultraviolet spectrometry. The purest fraction yielded ~50 μmol of ferric enterobactin.

Transport Experiments—An overnight culture in L broth of the strain to be studied was diluted 1:40 in Tris medium supplemented with glucose and incubated with shaking until the required absorbance at 600 nm was reached. Bacterial cells were harvested by centrifugation, washed with Tris medium without phosphate, and resuspended in phosphate-free Tris medium supplemented with glucose, and kept on ice until use. The transport medium was Tris medium containing 40–50 μM dihydrorhodanin (ligand/iron ratio of 4:1:3). Cells were grown for an additional 30, 60, and 120 min. At 0 min and each additional time, cells were cooled to 4 °C within 2 min, harvested, washed in Tris medium, and transferred to Delrin Mössbauer sample holders. All sample volumes were ~1 ml. Sample thickness did not exceed 9 mm. The containers were quickly frozen in liquid nitrogen and kept in a liquid nitrogen storage vessel until measurements were carried out. The Mössbauer spectra were recorded in the horizontal transmission geometry using a constant acceleration spectrometer operated in conjunction with a 512-channel spectrometer, recorded in the horizontal transmission geometry using a constant acceleration spectrometer operated in conjunction with a 512-channel spectrometer, and the radioactivity was measured at 10 °C.

Mössbauer Measurements—For each Mössbauer measurement, a 2-ml bacterial culture in 5-ml Erlenmeyer flasks was required to obtain ~1 ml of packed cells. Cultures of strains L2 cbsE-1 and L2 cbsH-19 were grown in Tris medium supplemented with glucose for 12 h. The absorbance at 600 nm was 0.65. 57Co-Labeled chrysobactin was added to the cell suspensions at a final concentration of 1.3 μM (lidan/iron ratio of 4:1.3). Cells were grown for an additional 30, 60, and 120 min. At 0 min and each additional time, cells were cooled to 4 °C within 2 min, harvested, washed in Tris medium, and transferred to Delrin Mössbauer sample holders. All sample volumes were ~1 ml. Sample thickness did not exceed 9 mm. The containers were quickly frozen in liquid nitrogen and kept in a liquid nitrogen storage vessel until measurements were carried out. The Mössbauer spectra were recorded in the horizontal transmission geometry using a constant acceleration spectrometer operated in conjunction with a 512-channel analyzer in the time-scale mode. The source was at room temperature and consisted of 1.15 GBq of 57Co diffused in rhodium foil (AEA, Amersham Biosciences AB). For transport experiments, the bacterial suspension was diluted in transport medium to give A600nm = 0.4 in a total volume of 5 ml and placed in a 50-ml Erlenmeyer flask. At intervals of 5–30 min, 200 μl was withdrawn and immediately filtered through a filter with 0.45-micron pores that had been soaked for at least 12 h in Tris medium supplemented with 20 μM unlabeled FeCl3. Filters were immediately washed with 20 ml of Tris medium without phosphate. The filters were placed in scintillation vials and air-dried, and radioactivity was measured. Two-μl samples of each bacterial culture were counted to check the total amount of radioactivity. For each strain, experiments were performed in triplicate.

Sequence analysis predicted a potential P promoter, overlapping the P′ promoter of the fct-cbsCEBA operon characterized previously (Fig. 2B). To determine the transcriptional start site of the cbsH gene, total RNA from iron-replete and -depleted cultures of E. chrysanthemi strain 3937 and E. coli strain JM101 harboring pCS1 was used as a template in extension reactions primed with a 32P-labeled 17-mer oligonucleotide complementary to the sequence between positions 158 and 142. For both strains, the reactions yielded iron-regulated CDNs that comigrated with an A residue (Fig. 2C). The occurrence of a transcriptional start at a T nucleotide (position 49) is consistent with the predicted P promoter. Two putative Fur-binding sites overlapping the −10/−35 sequences of the P promoter (Fig. 2B) account for the observed iron regulation. Regulation by iron was confirmed by monitoring expression of the chromosomal cbsH:17-lacZ fusion constructed in L2 cells (Table I and below) grown in Tris medium with and without iron supplementation (Fig. 2C).

The two potential translational start codons ATG1 and ATG2 (Fig. 2B) have good matches with the Shine-Dalgarno sequence (5′-GGAGG-3′ and 5′-GGACG-3′, respectively). To determine which of these codons is functional, we analyzed the translation products of two constructs (pCS2 and pLR1) placed under the control of the T7 φ10 promoter by SDS-PAGE. pCS3, which contains the 2.1-kb DraI-SalI fragment (Fig. 2A), includes both ATG codons and the 5′-untranslated region, pLR1, in which the 158-bp SspI-EcoNI fragment present in pCS3 has been deleted (Fig. 2, A and B), lacks the ATG1 codon. For both constructs, the same polypeptide migrating in the 43,000-Da range and induced at 42 °C only was identified (data not shown). Thus, under the conditions described, ATG2 (position 277) (Fig. 2B) is the functional translational start codon for the cbsH gene.

A cbsH Mutation Has No Effect on Iron Acquisition from Enterobactin in E. chrysanthemi—We investigated the protein encoded by the cbsH gene by isolating mutants (L2 cbsH-17 and L2 cbsH-19) (Table I) using insertional mutagenesis. Insertion cbsH-19 was mapped to position 943 by sequencing and was analyzed further. As the MuI1754 prophage generates polar mutations, we first investigated whether the mutant was able to produce chrysobactin. It did not grow on EDDHA/Ag agar medium. It produced catechol compounds, but did not release a functional siderophore, as shown by the chrome azurol S assay and growth stimulation experiments (data not shown). The introduction of pCS2, which carries the cbsH gene and ORF2, did not enable the mutant cells to grow on EDDHA/Ag agar medium. We therefore concluded that the mutant did not synthesize chrysobactin because of the polar effect on the downstream gene that shares identity with the E. coli entF gene.

As ferric enterobactin esterase is an essential component of the enterobactin-mediated iron transport pathway in E. coli, we investigated whether the L2 cbsH-19 mutant could use ferric enterobactin as an iron source (Table II). Ferric enterobactin promoted the growth of this mutant as efficiently as for a cbsH-positive strain. This mutant may be able to utilize ferric enterobactin because it produced a chromobactin or catechol. We therefore transduced the mutant with mutations acs-37 and cbsE-1. The transductant L2 cbsH-19 acs-37 cbsE-1 utilized ferric enterobactin as efficiently as did the simple mutant (Table II). This shows that a functional cbsH gene is not required in E. chrysanthemi for iron acquisition from enterobactin.
Lack of Functional Complementation of the E. coli fes Mutation by the cbsH Gene—These data led us to verify whether the cbsH gene could functionally complement an E. coli fes mutant. MM272-60 cells were transformed with plasmid pCS2 or pLR2. The transformants did not grow on EDDHA/L agar medium, and their growth was not stimulated by ferric enterobactin (Table II). A similar result was obtained with MM272-60 cells harboring pTF12, which contains the cbsH gene on a low-copy-number vector (Table II). We checked that the lack of complementation did not result from the production of nonphysiological levels of the CbsH protein by testing low-iron cultures of cells harboring pLR2 for the presence of enterobactin. All culture supernatants tested were strongly positive in the bioassay (data not shown).

A cbsH Mutation Affects Iron Acquisition from Chrysobactin in E. chrysanthemi—To determine whether the CbsH protein is a component of the chrysobactin-dependent iron transport pathway, we assessed the stimulation of growth of the L2 cbsH-19 acs-37 mutant by chrysobactin. After 24 h of incubation, the mutant had not grown; but after 72 h, a halo of growth became visible (Table II). The introduction of pCS2 into the mutant restored its growth in 24 h. Rescue was also observed after the introduction of pLR2, which carries the only cbsH gene (Table II). Thus, the mutant phenotype did not result from a polar effect of the mutation on downstream genes of the same operon. In contrast, the E. coli fes mutant (MM272-60), in which the ferric chrysobactin receptor fct gene is present on plasmid pLR3 (unlike pLR2), grew normally if supplied with ferric chrysobactin as an iron source (Table II). The halo of growth was similar with strain MM272-60 carrying pTF12, which contains the cbsH gene (Table II). Thus, the protein encoded by the cbsH gene is not required in E. coli cells if ferric chrysobactin is the iron source. One possible interpretation of these data is that a cbsH-negative mutant of E. chrysanthemi was affected in the transport of ferric chrysobactin.

We therefore determined the ability of the mutant to trans-
Bacterial strains, bacteriophages, and plasmids used

| Strain/plasmid | Relevant characteristics | Source/Ref. |
|---------------|--------------------------|-------------|
| E. chrysanthemi | Wild type isolated from African violet | Our collection |
| L2, L37 | Lac derivatives of 3937 | 63 |
| L37 acsA-1 fur | acsA-1 : MudI1734, fur- : KmR, SpecR, Acs- (achromobactin synthesis-deficient) Fur- | 32 |
| 3937 cbsE-1 | cbsE- : KmR, SpecR, Cbs- (chrysobactin synthesis-deficient) | 37 |
| L2 fct-34 | fct-34 : lacZ, fct- : Tn5-B20, KmR | 64 |
| L2 cbsH-17 | cbsH-17 : lacZ, cbsH : MudI1734, KmR, CbsH-, Cbs- | This work |
| L2 cbsH-19 | cbsH-19 : MudI1734, KmR, CbsH-, Cbs- | This work |
| L2 acs-37 | acs- : MudI11pR13, Acs-, KmR | 36 |
| L2 cbsE-1 | Cbs-, SpecR | This work |
| L2 acs-37 cbsE-1 | Acs-, Cbs-, KmR, CbsR, SpecR | This work |
| L2 cbsH-19 acs-37 cbsE-1 | Acs-, Cbs-, KmR, CbsR, SpecR | This work |
| L2 cbsH : aphA-3 acs-37 | CbsH-, Acs-, KmR, CmR | This work |
| E. coli K12 | supE hadΔ5 thiΔlac-proAB F [traD36 proABlacIq lacZΔM15] | 42 |
| TG1 | supE thiΔlac-proAB F [traD36 proABlacIq lacZΔM15] | 65 |
| JM109 | recA1 supE44 endA1 hadR17 gyrA96 relA1 thiΔlac-proAB F [traD36 proABlacIq lacZΔM15] | 65 |
| M8820 | F lacaraD139Δ ara-leu7697 Δ[proAB-argFlacIPOZYAKIII rpsL, SmR] | 66 |
| POI1734 | F MudI1734 (lac, KmR) ara- (muta33) DproAB-argFlacIPOZYAKIII rpsL, SmR | 66 |
| RW193 | F entA thi trpE proc leuB lacY mtl ygiK ara rpsL azi tss supE | T. Pugsley |
| RW1818–60 | F entA fepA thi trpE proc leuB lacY mtl ygiK ara rpsL azi tss supE | M. McIntosh |
| MM272–60 | F fes thi trpE proc leuB lacY mtl ygiK ara rpsL azi tss supE recA | 25 |
| BZB1013 | F fepA thyA38 depC2 IN1 | T. Pugsley |

Phage

ϕEC2 | Generalized transducing phage from E. chrysanthemi strain 3690 | 67 |

Plasmid

pUC19 | 2.7-kb vector, AmpR | 68 |
| pT7.6 | pT7.1 derivative, ApR | 42 |
| pWSK29 | pSC101 derivative, AmpR | 69 |
| pUC18K | 850-bp aphA-3 cassette in Smal site of pUC18, KmR, AmpR | 39 |
| pTF12 | 8.1-kb PetI-EcoRI fragment in pBR322, TcR | 23 |
| p248.34 | pT7F6 derivative with fct34 : Tn5-B20, TcR, KmR | 64 |
| pDE34 | 6.1-kb HindIII-SalI fragment from pTF6.34 in pUC18, ApR | 18 |
| pCS1 | Truncated derivative from SalI site of pDE34 (6 kb) | This work |
| pCS2 | 1.9-kb HpaI-BamHI fragment from pDE34 cloned into BamHI-HindIII sites of pUC19 | This work |
| pCS3 | 2.1-kb DraI-EcoRI fragment from pDE34 cloned into pT7.6 | This work |
| pLR1 | Derivative of pCS3 with 160-bp SstI-EcoNI deletion | This work |
| pLR2 | Derivative of pCS2 with 545-bp SacII-BamHI deletion | This work |
| pLR3 | 3.9-kb EcoRI-EcoNI fragment from pTF6.34 cloned into pWSK29 | This work |
| pLR4 | 850-bp aphA-3 cassette from pUC18K cloned into pCS2 | This work |

Iron source (μM DHB)

| Strain and genotype | Enterobactin (50 μM, 24 h) | Chrysobactin (50 μM) |
|---------------------|-----------------------------|---------------------|
|                      | 24 h                        | >72 h               |
| E. chrysanthemi L2  | fct-34 | ++ | − | − |
|                     | acs-37 cbsE-1 | ++ | ++ | ++ |
|                     | cbsH-19 | ++ | ++ | ++ |
|                     | cbsH-19 acs-37 cbsE-1 | ++ | ++ | ++ |
|                     | cbsH-19 aphA-3 cbs-37 | ++ | ++ | ++ |
|                     | cbsH-19 aphA-3 cbs-37 pLR2 | ++ | ++ | ++ |
| E. coli MM272–60 | fes | − | − | − |
|                    | fes pCS2 | − | − | − |
|                    | fes pLR2 | − | − | − |
|                    | fes pLR3 | − | − | − |
|                    | fes pTF12 | − | − | − |

TABLE II

Stimulation of the growth of E. chrysanthemi and E. coli mutants by enterobactin and chrysobactin

The growth of the mutants on EDDHA/L agar medium was scored in the presence of 10 μl of enterobactin or chrysobactin corresponding to 50 μM dihydroxybenzoic acid (DHB) equivalents as described under “Experimental Procedures.” Diameters of growth zones (in millimeters) are indicated as follows: ++, 18 ± 2; +++, 24 ± 2; +, no growth. Experiments were carried out at least in duplicate.

| Strain and genotype | Iron source (μM DHB) |
|---------------------|---------------------|
|                     | Enterobactin (50 μM) | Chrysobactin (50 μM) |
|                     | 24 h | >72 h |
| E. chrysanthemi L2 | fct-34 | ++ | − | − |
|                     | acs-37 cbsE-1 | ++ | ++ | ++ |
|                     | cbsH-19 | ++ | ++ | ++ |
|                     | cbsH-19 acs-37 cbsE-1 | ++ | ++ | ++ |
|                     | cbsH-19 aphA-3 cbs-37 | ++ | ++ | ++ |
|                     | cbsH-19 aphA-3 cbs-37 pLR2 | ++ | ++ | ++ |
| E. coli MM272–60 | fes | − | − | − |
|                    | fes pCS2 | − | − | − |
|                    | fes pLR2 | − | − | − |
|                    | fes pLR3 | − | − | − |
|                    | fes pTF12 | − | − | − |

port. 59Fe-labeled chrysobactin and compared it with that of the parental strain (Fig. 3). Strains were grown in Tris medium, and uptake experiments were conducted with cells harvested at A600nm = 0.6. After 5 h, the growth of mutant L2 cbsH-19 acs-37 had slowed considerably, indicating that iron was poorly assimilated (Fig. 3A). The ferric chrysobactin transport rate was higher in the mutant than in the parental strain (Fig. 3B). Thus, the mutation had no effect on ferric chrysobactin transport per se. The transport rate seems to depend on the intracellular metabolic state and presumably reflects the level of derepression by iron of the entire protein machinery involved in transport.

Accumulation of Ferric Chrysobactin in a Nonpolar cbsH-negative Mutant—We investigated the protein encoded by the cbsH gene by constructing a nonpolar mutant with the aphA-3 cassette (39). Mutant L2 cbsH : aphA-3 acs-37 gave rise to colonies with a red color that was not observed in polar mutants (Table II). This mutant grew very slowly in Tris medium, but transported 59Fe-labeled chrysobactin very quickly, indicating that the bacterial cells were severely iron-depleted (Fig. 3B). These observations suggest that an iron-binding compound accumulated inside the cells. To determine whether this compound was the ferric chrysobactin complex, cell extracts of L2 cbsH : aphA-3 acs-37 were compared with those of a fur mutant (L37 acsA-1 fur) that also overexpresses chrysobactin biosynthesis and transport proteins in Tris medium supplemented with FeCl3. Bioassay showed that extracts from cbsH-negative cells promoted the
The CbsH Protein Is a Peptidase Hydrolyzing Chrysobactin—

The accumulation of the ferric chrysobactin complex in the cytosol of cbsH-negative cells indicates that this molecule was not degraded following its transport. As chrysobactin possesses a peptide bond, one possibility was that the cbsH gene encodes a peptidase. Indeed, the catalytic mechanism of certain esterases involves the formation of an acetyl-enzyme intermediate during the reaction that is analogous to that of serine proteases (47). The alignment of ferric enterobactin esterase-like protein sequences from various bacterial genera present in data banks (Fig. 5) reveals the presence of a common signature, GXXXG-GDGH found in the family of prolyl oligopeptidases (48). These residues are within ~130 residues of the C terminus, and the N-terminal parts of the molecule are more or less variable. Therefore, we investigated whether cbsH-positive cells had an enzyme enabling them to catalyze the hydrolysis of ferric chrysobactin that was lacking in mutant cells. Enzymatic activity was determined in cell extracts from low-iron cultures of the parental strain L2 cbsE-1 and the mutant L2 cbsH-19 (Table III). We observed the disappearance of the ferric chrysobactin complex only in cbsH-positive cells. This enzymatic activity was thioldependent, like a number of other cytosolic peptidases. The addition of diisopropyl fluorophosphate, an inhibitor of serine proteases, totally blocked the reaction. These results show that the CbsH protein has a ferric chrysobactin peptidase activity. To also determine whether this enzyme has a ferric enterobactin esterase activity, we used ferric enterobactin as a substrate. Although cbsH-positive cells had a significant level of ferric enterobactin esterase activity compared with mutant cells, the specific enzymatic activity was 10 times lower than that found for the hydrolysis of ferric chrysobactin under the same conditions.

Iron Removal from Chrysobactin in Vivo Does Not Require a Functional cbsH Gene—To obtain basic information on the metabolic utilization of chrysobactin-bound iron, in situ Mössbauer spectroscopy of whole cells was performed at various stages of chrysobactin uptake. In principle, in situ Mössbauer spectroscopy enables the simultaneous identification of all main iron metabolites at a qualitative as well as quantitative level without destruction of the cellular assembly (49, 50). Moreover, time-dependent changes can be followed, the resolution of which is merely limited by the time required for sample preparation (50).

As expected, samples of either strain (L2 cbsE-1 and L2 cbsH-19) taken directly after addition of 57Fe-labeled chrysobactin yielded Mössbauer spectra with very poor resolution. The cbsH-positive sample exhibited a single doublet of ferrous high-spin iron in an octahedral oxygen or nitrogen environment: δ = 1.26 (6) mm/s, ΔEq = 3.19 (11) mm/s, and Γ = 0.512 mm/s. This component accounts for most of the Mössbauer absorption (84%). Based on the evolution of a second component visible after growth with 57Fe-labeled chrysobactin, this second component was fitted to the following experimental data: δ1 = 0.38 (6) mm/s, ΔEq = 0.65 (5) mm/s, and Γ = 0.27 mm/s (16%). For the cbsH-negative strain, a featureless absorption was found. Nevertheless, we tried a fit of δ1 = 0.48 (8) mm/s, ΔEq = 0.65 (11) mm/s, and Γ = 0.7 mm/s (75%) and δ2 = 0.97 (6) mm/s, ΔEq = 1.54 (11) mm/s, and Γ = 0.27 mm/s. After 30, 60, and 120 min, the Mössbauer spectra were well resolved and allowed unambiguous analysis (Fig. 6 and Table IV). The Mössbauer parameters of the cbsH-positive strain and its mutant after 57Fe-labeled chrysobactin uptake are summarized in Table IV. Like other catechol-type siderophores (49, 51), chrysobactin exhibited a typical magnetically split S = 5/2 pattern (Fig. 7A). No ferric chrysobactin was detectable in Mössbauer spectra of whole cells. In contrast, there was almost exclusively
ferrous iron found at \( t = 0 \) (Fig. 6A and Table IV); and even after 30 min of uptake (Fig. 6B and Table IV), the majority of the transported iron was present in its ferrous form (Fig. 6B and Table IV).

The second component observed spectroscopically corresponds to a ferric high-spin species. The 57Fe content of the cells grew with increasing incubation time (increasing total absorption area), although it was slightly slower for the cbsH-positive strain. Whereas after 30 min of incubation the ferric iron species contributed only little to the Mössbauer absorption, it represented the major component after 2 h. This species exhibited Mössbauer parameters very similar to those of bacterioferritin found in \( E. coli \) (54, 55). Comparison of the Mössbauer parameters obtained from a spectrum measured at 86 K (data not shown) with those derived from a spectrum at 4.3 K (Fig. 6) revealed a significant increase in \( \gamma \)-factor (from 0.505 to 0.814 mm/s) and a concomitant decrease in relative transmission. This considerable line broadening is typically found at temperatures close to superparamagnetic transitions (53, 54).

\( E. coli \) -

TABLE III

| Strain      | FeCb hydrolyzed | FeEnt hydrolyzed |
|-------------|----------------|-----------------|
| L2 cbsE-1   | 29.4 (3.64)    | 2.30 (0.75)     |
| L2 cbsH-19  | 1.03 (0.6)     | 0.64 (0.33)     |

Fig. 5. Multiple alignment of Fes-related proteins from various bacterial species. Asterisks indicate identical amino acids, and dots indicate residues with similar chemical properties. Boldface letters with asterisks correspond to amino acids conserved in the S9 prolyl oligopeptidase family (48). His and Asp residues in boldface can potentially belong to the catalytic site.

\( E. chrysanthemi \) Chrysobactin Peptidase

\( 2391 \)
type bacterioferritins display magnetic broadening below 4.3 K and eventually show magnetically split spectra at temperatures below 1 K (56). Fig. 7B displays the Mössbauer spectrum of cbsH-negative mutant cells measured at 1.8 K. Indeed, the ferric iron species is missing in this spectrum; instead, a magnetically broadened absorption is visible, as expected for a bacterioferritin-type protein. Therefore, we attribute the ferric iron species to a bacterioferritin-like compound.

DISCUSSION

In this study, we report the functional analysis of a new gene (cbsH) that belongs to the chrysobactin-dependent iron transport gene cluster of E. chrysanthemi strain 3937. The cbsH gene is the first gene of an operon involved in chrysobactin biosynthesis and transcribed from the iron-regulated divergent promoter fct-cbsH, which controls, in the opposite orientation, the transcription of the fct-cbsCEBA operon, identified earlier (23). The cbsH gene is 64% identical to the E. coli fes gene for the 871 nucleotides from positions 272 to 1143. It encodes a polypeptide with an apparent molecular mass of 43,000 Da, a size similar to that reported for the E. coli Fes protein (25). The CbsH protein is 45.6% identical to the Fes protein of E. coli, 46% identical to the Fes protein of Yersinia enterocolitica (57), and 42% identical to Salmonella enterica IroD (iroD gene, GenBank™/EBI Data Bank accession number U97227), with the level of identity uniform over the entire amino acid sequence (according to the program BLAST).

The presence in E. chrysanthemi of a homolog of the ferric enterobactin esterase of E. coli was expected. E. chrysanthemi has a ferric enterobactin transport system that supplies the cell with iron. As the hydrolysis of ferric enterobactin is essential in E. coli cells, we thought it likely that this molecule would have the same fate in E. chrysanthemi. We have shown that the CbsH protein is not required for the removal of iron from ferric enterobactin in E. chrysanthemi. These data indicate that
cleavage of the ester bonds of ferric enterobactin is not required in *E. chrysanthemi* for iron reduction and release. This was not because of the presence of an additional *fes*-like gene on the *E. chrysanthemi* chromosome, as shown by DNA/DNA hybridization analysis (data not shown). In contrast, the *fes* homolog from *Y. enterocolitica* appears to be absolutely required for ferric enterobactin utilization in this bacterium (57). In addition, the *viuB* gene from *Vibrio cholerae*, which is involved in vibriobactin processing, can complement the *E. coli* *fes* mutation (58). No functional complementation of the *E. coli* *fes* mutation was observed with the *E. chrysanthemi* *cbsH* gene.

These results show that iron release from enterobactin is not *CbsH*-dependent. Instead, a *Fes/CbsH*-independent mechanism has to be considered.

We should point out that the role of the *E. coli* ferric enterobactin esterase has been much debated. In particular, several experimental aspects have remained unexplained (10, 11, 59). For instance, this enzyme is required for the removal of iron from enterobactin analogs devoid of ester bonds (60, 61). The redox potential of a ferric siderophore depends on the binding constant for iron and thus on the capacity of the molecule to be protonated at neutral pH (62). If the internal pH of *E. chrysanthemi* were slightly lower than that of *E. coli*, then iron would be easier to extract in *E. chrysanthemi* than in *E. coli*. Cohen et al. (59) have reported that enterobactin, like synthetic analogs such as TRENSAM, may adopt a Tris salicylate mode of binding if sequentially protonated, with iron release facilitated by a biological reductant.

On the basis of amino acid sequence comparisons, we found that the CbsH protein displays characteristics of the S9 prolyl oligopeptidase family (48), viz. the conservation of amino acids around the catalytic triad Ser, Asp, and His (Fig. 5). The S9 family contains serine peptidases with a varied range of restricted specificities, including oligopeptidase B from eubacteria, which cleaves arginyl and lysyl bonds. In agreement with sequence predictions, we showed that the CbsH protein is an enzyme able to degrade ferric chrysobactin in the cytosol. This hydrolytic activity is thiol-dependent and inhibited by fluorophosphates such as diisopropyl fluorophosphate. Given the chrysobactin structure (Fig. 1), it is very likely that this enzyme cleaves the lysyl bond, thus forming 2,3-dihydroxybenzoyllysinine and serine.

To further understand the role of this enzyme, we analyzed the cellular distribution and reoxid state of iron following the transport of ferric chrysobactin into the cytosol using Mössbauer spectroscopy. After 30 min of incubation with 57Fe-labeled chrysobactin, the Mössbauer spectra of the *cbsH* mutant showed mainly ferrous iron, which is involved in iron transport of *E. chrysanthemi* (56). The absorption areas were slightly lower than that of the *cbsH* strain and of a *cbsH*-negative mutant cells measured at 1.8 K (B). The redox potential of a ferric siderophore depends on the binding constant for iron and thus on the capacity of the molecule to be protonated at neutral pH (62).

The Mössbauer parameters and the corresponding percentage of the absorption areas are listed in Table IV (Parts A and B).

### Table IV

| Strain and genotype | δ  | Δ  | Γ  | Relative area | Time |
|--------------------|----|----|----|---------------|------|
|                    | mm/s | mm/s | mm/s | % | min |
| A                  |     |     |     |   |     |
| *cbsH*             | 0.48 | 0.65 | 0.58 | 13 | 0   |
| *cbsH*             | 0.42 | 0.72 | 0.75 | 70 | 0   |
| *cbsH*             | 0.48 | 0.65 | 0.59 | 22 | 30  |
| *cbsH*             | 0.50 | 0.71 | 0.57 | 24 | 30  |
| *cbsH*             | 0.47 | 0.65 | 0.46 | 61 | 60  |
| *cbsH*             | 0.49 | 0.69 | 0.78 | 53 | 60  |
| *cbsH*             | 0.49 | 0.708 | 0.66 | 64 | 120 |
| *cbsH*             | 0.50 | 0.68 | 0.5 | 78 | 120 |
| B                  |     |     |     |   |     |
| *cbsH*             | 1.28 | 3.14 | 0.58 | 87 | 0   |
| *cbsH*             | 0.96 (?) | 1.54 (?) | 0.39 (?) | 30 | 0   |
| *cbsH*             | 1.25 | 3.01 | 0.64 | 78 | 30  |
| *cbsH*             | 1.26 | 3.02 | 0.71 | 76 | 30  |
| *cbsH*             | 1.20 | 2.86 | 0.55 | 39 | 60  |
| *cbsH*             | 1.27 | 3.03 | 0.72 | 47 | 60  |
| *cbsH*             | 1.22 | 3.04 | 0.76 | 35 | 120 |
| *cbsH*             | 1.25 | 2.95 | 0.52 | 22 | 120 |

**Fig. 7.** Mössbauer spectra of a frozen aqueous solution (Tris buffer (pH 7)) of 57Fe-labeled chrysobactin (1:4) measured at 4.3 K (A) and of frozen *E. chrysanthemi cbsH*-negative mutant cells measured at 1.8 K (B) in a field of 20 milliteslas perpendicular to the γ-rays. The cell suspension was supplied with 5 mM 57Fe-labeled chrysobactin at A\text{expt} = 0.65. Cells were harvested after 120 min of additional growth. The Mössbauer parameters and the corresponding relative absorption areas are listed in Table IV (Parts A and B).
mic membrane or in the cytosol. The affinity of catecholate siderophores for ferrous iron is very low. Even water is a better chelator of ferrous high-spin iron than these siderophores. Therefore, the presence of ferrous iron provides evidence for a rapid reductive release of the metal from its carrier, preventing an observable intracellular concentration of $^{57}$Fe-labeled chrysobactin. Although a ferrous hexaquo complex is stable in a strict reductive (and anaerobic) environment, it is very likely that the reduced metal is complexed by a specific intracellular chelator to prevent Haber-Weiss-Fenton chemistry (50). Previously, we found that ferrous iron constitutes one of the major cellular iron species in many microorganisms under conditions of siderophore-controlled growth (52, 53). The corresponding compound has been isolated from E. coli and from Pantoea agglomerans and partially characterized as an oligomeric sugar phosphate (52). It was termed ferrochelatin (53). Based on the previous studies, we attribute the detected ferrous iron to ferrochelatin. Where ferrochelatin-bound iron keeps its intracellular concentration at a certain level (~0.3%), the second component of the Mössbauer spectra increases its contribution by time and represents the major component after 2 h of incubation. Based on the temperature-dependent Mössbauer spectra and their parameters, the conclusion must be drawn that this component represents a bacterioferritin-like iron storage compound. Thus, ferrous iron released from chrysobactin is immediately transferred into the iron storage form, where it is oxidized again at the ferroxidase site (71, 72). In summary, the Mössbauer spectroscopic analysis shows significant differences neither in the chrysobactin-mediated iron uptake between the parental strain and its mutant nor in the metabolic distribution pattern. Based on the results of this investigation, it is safe to state that metabolic utilization of both enterobactin- and chrysobactin-bound iron is not cbsH-dependent (see scheme shown in Fig. 8).

As described above, there is good evidence for hydrolytic cleavage of chrysobactin by CbsH. In addition, long-term growth inhibition has been observed in cbsH-negative mutants. This ligand hydrolysis occurs obviously after iron removal, and lack of hydrolysis in the mutants results in growth inhibition. This unexpected finding might be linked either to utilization of the aromatic systems of chrysobactin for anabolic reactions or to a role of cbsH in intracellular iron homeostasis. Within the rationale of bacterial iron metabolism, we favor the latter line of thought. The free chrysobactin ligand is thermodynamically capable of extracting ferrous iron from all intracellular ferric iron sources exhibiting a lower complex formation constant than that of ferric chrysobactin. In addition, recent studies on the uptake of iron(III) by chrysobactin have shown that the carboxyl group of the serine residue in chrysobactin strongly influences the kinetics of formation of the ferric complex. To prevent iron removal from metabolically active enzymes or from any accessible intracellular iron pool, either the ligand has to be re-excreted (which is known for some bacterial siderophore uptake systems), or it must be degraded or modified. At this point, it is important to note that the nonpolar cbsH-negative mutant behaves as if it was severely iron-depleted, although it contains high levels of ferric chrysobactin. This finding fits well with our hypothesis because an intracellular post-transport recomplexation of iron by the non-degraded ligand seems to occur. In summary, taking all pieces of circumstantial evidence together, we suggest that hydrolytic degradation of chrysobactin by CbsH is aimed at keeping the intracellular iron distribution at a well regulated level (iron homeostasis) in E. chrysanthemi strain 3937.

Acknowledgments—We thank Céline Masciaux and Chrystèle Sauvage for the construction of recombinant plasmids and interest in this work. Prof. Kenneth Raymond and Thierry Franza for helpful discussions, Dr. Anne-Marie Albrecht-Gary for communicating data prior to publication, and Alex Edelman for reading the manuscript.

REFERENCES

1. Braun, V., Hantke, K., and Koster W. (1998) in Metal Ions in Biological Systems: Iron Transport and Storage in Microorganisms, Plants and Animals (Sigel, A., and Sigel, H., eds) Vol. 35, pp. 67–145, Marcel Dekker, Inc., New York
2. Postle, K. (1999) Nat. Struct. Biol. 6, 3–6
3. Matzanke, B. F., Muller-Matzanke, G., and Raymond, K. N. (1989) in Iron Carriers and Iron Proteins (Locher, T. M., ed) Vol. 5, pp. 89–109, VCH Publishers, Inc., New York
4. Fontecave, M., Coves, J., and Pierre, J.-L. L. (1994) BioMetals 7, 3–8
5. Lodge, J. S., Gaines, C. G., Arceneaux, J. E. L., and Byers, B. R. (1980) Biochim. Biophys. Acta 589, 1–15
6. Fischer, E., Strebol, B., Hartz, D., and Braun, V. (1990) Arch. Microbiol. 153, 329–336
7. Coves, J. and Fontecave, M. (1993) Eur. J. Biochem. 211, 635–641
8. Halle, F., and Meyer, J.-M. (1992) Eur. J. Biochem. 209, 621–627
9. O'Brien, G., and Gibson, F. (1970) Biochim. Biophys. Acta 215, 393–402
10. Greenwood, K. T., and Luke, R. K. J. (1978) Biochim. Biophys. Acta 525, 269–288
11. Brickman, T. J., and McIntosh, M. A. (1992) J. Biol. Chem. 267, 12350–12355
12. Winkelmann, G., Cansier, A., Beck, W., and Jung, G. (1994) BioMetals 7, 139–154
13. Brickman, T. J., and McIntosh, M. A. (1993) J. Biol. Chem. 268, 12848–12855
14. Porra, R. J., Langman, L., Young, I. G., and Gibson, F. (1972) Arch. Biochem. Biophys. 153, 74–78
15. Expert, D. (1999) Annu. Rev. Phytopathol. 37, 307–334
16. Persmark, M., Expert, D., and Neillands, J. B. (1989) J. Biol. Chem. 264, 1387–1393
17. Persmark, M., Expert, D., and Neillands, J. B. (1992) J. Biol. Chem. 267, 4783–4789
18. Sauvage, C., Franz, T., and Expert, D. (1996) J. Bacteriol. 178, 1227–1231
19. Enard, C., and Expert, D. (2000) Microbiology 146, 2051–2056
20. Enard, C., Dolez, A., and Expert, D. (1988) J. Bacteriol. 170, 2419–2426
21. Enard, C., Franz, T., Neema, C., Gill, P. E., Persmark, M., Neillands, J. B., and Expert, D. (1991) Plant Soil 130, 263–271
22. Munzing, M., Bukiwicziez, H., Expert, D., Enard, C., and Meyer, J.-M. (2000) Z. Naturforsch. 55C, 328–332
23. Franz, T., and Expert, D. (1991) J. Bacteriol. 173, 6874–6881
24. Hunt, M. D., Pettis, G. S., and McIntosh, M. A. (1994) J. Bacteriol. 176, 3944–3955
25. Pettis, G. S., Brickman, T. J., and McIntosh, M. A. (1968) J. Biol. Chem. 263, 18857–18863
26. Lundrigan, M. D., and Kadner, R. J. (1986) J. Bacteriol. 161, 10797–10801
27. Armstrong, S. K., Francis, C. L., and McIntosh, M. A. (1990) J. Bacteriol. 166, 1452–1454
28. Pettis, G. S., and McIntosh, M. A. (1987) J. Bacteriol. 169, 4154–4162
29. Coderre, P. E., and Earhart, C. F. (1989) J. Gen. Microbiol. 135, 3043–3055
30. Reuzeau, F., Sakaitani, M., Drueckhammer, D., Reichert, J., and Walsh, C. T. (1991) Biochemistry 30, 2916–2927
31. Gehring, A. M., Mori, I., and Walsh, C. T. (1998) Biochemistry 37, 2646–2659
32. Franz, T., Sauvage, C., and Expert, D. (1999) Mol. Plant Microbe Interact. 12, 119–129
33. de Lorenzo, V., Giovannini, F., Herrera, M., and Neillands, J. B. (1988) J. Biol. Chem. 263, 857–864
34. Escolar, L., Perez-Martín, J., and de Lorenzo, V. (1999) J. Bacteriol. 181, 6223–6229
35. Franz, T., Enard, C., Van Gijsegem, F., and Expert, D. (1991) Mol. Microbiol. 5, 1319–1329
36. Expert, D., Sauvage, C., and Neillands, J. B. (1992) Mol. Microbiol. 6, 2009–2017
37. Malhe, B., Maslax, C., Rauscher, L., Enard, C., and Expert, D. (1995) Mol. Microbiol. 18, 34–43
38. Miller, J. F. (1972) Experiments in Molecular Genetics, pp. 431–435, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
39. Ménard, R., Sansonetti, P., and Faurès, C. (1993) J. Bacteriol. 175, 5899–5906

---

$^{2}$ B. F. Matzanke, unpublished data.

$^{3}$ A.-M. Albrecht-Gary, personal communication.
