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Paracrystalline Inclusions of a Novel Ferritin Containing Nonheme Iron, Produced by the Human Gastric Pathogen *Helicobacter pylori*: Evidence for a Third Class of Ferritins

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An abundant 19.3-kDa *Helicobacter pylori* protein has been cloned, and the sequence is homologous with a ferritin-like protein produced by *Escherichia coli* K-12. Homologies are also present with a number of eucaryotic ferritins, as well as with the heme group-containing bacterioferritins. All amino acids involved in chelation of inorganic iron by ferritins from humans and other higher species are conserved in the *H. pylori* protein. Consistent with the structural data indicating an iron-binding function, *E. coli* overexpressing the *H. pylori* ferritin-like protein accumulates almost 10 times more nonheme iron than vector controls, and the iron-binding activity copurifies with the 19.3-kDa protein. Immunoelectron microscopy of *H. pylori*, as well as of *E. coli* overexpressing the *H. pylori* gene, demonstrates that the gene product has a cytoplasmic location where it forms paracrystalline inclusions. On the basis of these structural and functional data, we propose that the *H. pylori* gene product (termed Pfr) forms the basis for a second class of bacterial ferritins designed to store nonheme iron.

A considerable wealth of information on how bacteria scavenge iron and the important role these different iron uptake systems have in microbial pathogenesis is available (4). In contrast, little is known about the fate of iron once inside the microorganism and about the mechanism by which the organism protects itself against iron toxicity (3). In eucaryotic organisms excess nonheme bound iron can be stored by ferritin. Eucaryotic ferritins are multimeric complexes composed of 24 subunits, each with a monomer molecular mass of ≈20,000 Da; each ferritin shell may enclose up to 4,500 iron atoms (6, 28). In bacteria, bacterioferritin (Bfr), also called cytochrome *b* because of its content of heme, has been found in microorganisms such as *Azotobacter vinelandii* (26), *Escherichia coli* (30), *Pseudomonas aeruginosa* (21), and *Nitrobacter winogradskyi* (16). Even though the sequence homologies between Bfr's and eucaryotic ferritins are low, careful alignments show that key residues important for the structure and function of eucaryotic ferritins are also present in the bacterioferritins (1, 11).

Helicobacter pylori is a microaerophilic, spiral, gram-negative bacterium that has the human gastric mucosa as its exclusive habitat (29). *H. pylori* has been implicated as a causative agent in chronic active (type B) gastritis (13), gastric and duodenal ulcers (9, 10), and gastric adenocarcinoma (22, 23). We have cloned and sequenced a gene from *H. pylori* that encodes a cytosolic protein that is predicted to have significant homology with the eucaryotic ferritins and Bfr's and that forms paracrystalline inclusion bodies in the bacterial cytosol. Consistent with the structural predictions, the protein is shown functionally to bind inorganic iron. Since *E. coli* expresses a protein similar to that of *H. pylori* (15) and since the two proteins show similar identity to eucaryotic ferritins, it is proposed that the procaryotic ferritins (Pfr) reported here form a second class of bacterial ferritins designed to store nonheme iron in a specialized cytosolic compartment protecting the cells from iron toxicity.

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MATERIALS AND METHODS

Cloning of *pfr*. Loosely associated aggregates from *H. pylori* NCTC 11638 were released by vortexing, and this material mediated hemagglutination in a manner similar to that of whole cells when incubated with human A2 erythrocytes (data not shown). In an attempt to characterize the hemagglutinin present in this extract, an antiserum was raised in rabbits against a fetuin-agarose (Sigma, St. Louis, Mo.)-absorbed fraction of the extract. Although the antiserum recognized a number of proteins in the *H. pylori* extract, the major reactivity in immunoblots was against a polypeptide with a molecular mass of about 20 kDa (data not shown). The proteins migrating between 19 and 20 kDa were electroblotted onto a polyvinylidene difluoride membrane (20), and the amino-terminal sequence was determined by sequential Edman degradation. The primary sequence obtained was back translated into nucleotides, and degenerate oligonucleotide primers were synthesized. By using these primers on chromosomal DNA from *H. pylori* NCTC 11638, a 60-bp fragment was generated by polymerase chain reaction amplification. This fragment was cloned into the pCR2000 TA cloning vector (Invitrogen, San Diego, Calif.), and its nucleotide sequence was determined. Translation of the nucleotide sequence revealed one frame that corresponded exactly to the amino-terminal sequence obtained

from the electroblotted protein. Next, the 60-bp fragment generated by polymerase chain reaction amplification was used to probe a cosmid library from *H. pylori* NCTC 11638. One cosmid clone that hybridized to the probe was identified. The nucleotide sequence of the *pfr* gene was determined by plasmid sequencing using *pfr*-specific primers and found to encode a polypeptide of 167 amino acids. The deduced amino acid sequence of the 5′ end of the cloned gene corresponded exactly to the amino-terminal sequence originally obtained from the gel-purified protein, indicating that this 19.3-kDa polypeptide was translated in the absence of a cleavable signal peptide.

Expression of Pfr in *E. coli*. The *pfr* gene was cloned into the vector pET3a under control of the bacteriophage T7 promoter and used in an inducible T7 expression system (27). Oligonucleotide primers that incorporated base changes necessary to produce *Nde*I and *Bam*HI restriction sites were used to polymerase chain reaction amplify the entire *pfr* gene. The polymerase chain reaction product DNA was then subcloned into the vector pET3a by using the *Nde*I and *Bam*HI sites (27). Correct insert orientation in the expression vector was confirmed by restriction enzyme analysis and partial DNA sequence analysis. *E. coli* BL21(DE3) was then transformed with pET3a-Pfr carrying the *pfr* gene and used for induced expression of the protein in Luria-Bertani medium (27). Protein levels were estimated by laser densitometric scanning (Ultrascan XL; Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) of Coomassie blue-stained gels and quantitated by comparison with parallel lanes containing known amounts of marker proteins.

Purification of Pfr. For purification of the *H. pylori* ferritin homolog expressed in *E. coli* BL21(DE3)(pET3a-Pfr), bacteria were grown as described above, washed, resuspended in 20 mM Tris buffer (pH 7.6), and lysed by sonication. Cell debris was cleared by centrifugation. The supernatant was filtered through a 0.2-μm-pore-size filter unit (Millipore) prior to fractionation by fast protein liquid chromatography (FPLC) as described elsewhere (5). Column fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue, and densitometric scanning demonstrated that this single anion-exchange chromatography step resulted in at least a threefold purification of the protein.

Iron and heme content assays. For both iron and heme determinations, bacteria were induced for protein expression in Luria-Bertani medium (27), washed three times in phosphate-buffered saline (PBS; pH 7.0), resuspended at 1.5 × 10^9/ml, frozen, quick thawed, and lysed by sonication. Cell debris was removed by centrifugation at 14,000 × *g* for 10 min. Iron was quantitated by the Barnes Hospital Clinical Chemistry Lab (St. Louis, Mo.) using the Kodak Ektachem method with a model E700XR analyzer. Type VI ferritin from rat liver (Sigma) served as the positive control (data not shown). The concentration of heme was measured by the pyridine hemochrome method (8) on a second aliquot of the samples prepared for iron determinations. Rat hemoglobin (Sigma) served as the positive control (data not shown).

Electron microscopy. Bacteria were fixed in 1% glutaraldehyde in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2)–30 mM NaCl–1 mM $MgCl_2$–0.5 mM $CaCl_2$ for 60 min at room temperature and processed for cryoimmunoelectron microscopy as previously described (25). Frozen thin sections were probed with a preabsorbed rabbit anti-Pfr antiserum, followed by 15-nm-gold-conjugated goat anti-rabbit immunoglobulin G (Amersham). The sections were then stained and embedded in 2% polyvinyl alcohol and 0.2% uranyl acetate and examined.

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CTTTCAAAGCATGTAATGCCTTCATTTAAGTAACATGTAGCATTTAGATGAGATGCTTTT   60

ATATATTATATAAAAATATCCCTTTTAATCCCCCCCTATTGATACCAGCCCCCTTTTTTT  120

GACCTAATTCTCATTAAAAGCACTTTTATGATAAAATCTAAGCTTTATCAAGCCATTAGC  180

TGGCGTTCTTTCTCATTTTTTGCAAGTTTTTAAAAATTTTCATACTCTTGTTTACTTTTT  240

CATTATCATTTATGCTATAATTATGGGACAACTTAAACCAACACAAGGAGATACTATGT  300
                                        S.D.      M  L

TATCAAAAGACATCATTAAGTTGCTAAACGAACAAGTGAATAAGGAAATGAACTCTTCCA  360
  S  K  D  I  I  K  L  L  N  E  Q  V  N  K  E  M  N  S  S  N

ACTTGTATATGAGCATGAGTTCTTGGTGCTATACCCATAGCTTAGACGGCTCGGGGCTTT  420
  L  Y  M  S  M  S  S  W  C  Y  T  H  S  L  D  G  S  G  L  F

TCTTGTTTGACCATGCGGCTGAAGAATACGAGCATGCTAAAAAGCTTATCGTCTTCTTGA  480
   L  F  D  H  A  A  E  E  Y  E  H  A  K  K  L  I  V  F  L  N

ATGAAAACAATGTGCCTGTGCAATTGACTAGCATCAGCGCGCCTGAGCATAAGTTTGAAG  540
   E  N  N  V  P  V  Q  L  T  S  I  S  A  P  E  H  K  F  E  G

GTTTGACTCAAATTTTCCAAAAAGCCTATGAACATGAGCAACACATCAGCGAGTCTATTA  600
   L  T  Q  I  F  Q  K  A  Y  E  H  E  Q  H  I  S  E  S  I  N

ATAATATCGTCGATCACGCCATAAAAGGCAAAGATCATGCGACTTTCAATTTCTTGCAAT  660
   N  I  V  D  H  A  I  K  G  K  D  H  A  T  F  N  F  L  Q  W

GGTATGTGTCTGAACAGCATGAAGAAGAAGTGCTTTTCAAGGATATTTTGGATAAAATTG  720
   Y  V  S  E  Q  H  E  E  E  V  L  F  K  D  I  L  D  K  I  E

AGTTGATTGGTAATGAAAACCATGGCTTGTATTTGGCTGATCAGTATGTCAAAGGGATCG  780
   L  I  G  N  E  N  H  G  L  Y  L  A  D  Q  Y  V  K  G  I  A

CTAAAAGCAGGAAATCTTAATTTTAGGGTCATTGAGTGCAAAAACTAGCCGTTTTTGATT  840
   K  S  R  K  S  *

TTGACTCCACGCTAGTCAATGCTGAGACGATTGAGTCTTTAGCGAGGGCGTGGGGGTGTT  900

TGATGAAGGTGAAACAATCACTTCACAAGCCATGAATGCAGACAGATTTCATAAAGTCTA  960

TTGAGGTTCACTAACGTCCGT                                          981
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FIG. 1. Nucleotide sequence of the *H. pylori* NCTC 11638 *pfr* gene coding for a procaryotic ferritin. The putative Shine-Dalgarno (S.D.) region is underlined. The deduced amino acid sequence is given. The N-terminal peptide sequence of the Pfr protein expressed in *H. pylori* as determined by Edman degradation is also underlined.

Antisera. All antisera were produced in rabbits according to standard protocols (12). For production of the antiserum against *H. pylori* Pfr, preparative SDS-PAGE was performed with whole-cell lysates of induced BL21(DE3)(pET3a-Pfr) bacteria and the region of the gel containing the Pfr protein was excised (see Fig. 3) and used for immunization. The resulting antiserum was absorbed against an acetone precipitate of BL21(DE3)(pET3a) vector control bacteria prior to use in immunoelectron microscopy.

RESULTS

Cloning and sequence analysis of *pfr*. *H. pylori* NCTC 11638 expresses a sialic acid-specific hemagglutinin. During our attempts to characterize the adhesin, we isolated, cloned, and sequenced the gene coding for a dominant 19.3-kDa protein (Fig. 1). A homology search using the Swiss protein data base (24) revealed that the *H. pylori* protein exhibited 42.3% identity to a 165-amino-acid *E. coli* protein previously demonstrated to show similarity to the human ferritin H subunit (1, 15). Comparison of the *H. pylori* 19.3-kDa protein with the entire Swiss protein data base by using the FASTDB computer program gave high homology scores for eucaryotic ferritins. The sequence identity between the *H. pylori* 19.3-kDa protein and eucaryotic ferritins ranged from 26.7% with *Xenopus laevis* ferritin to 20.1% with rabbit ferritin L chain. The *H. pylori* and *E. coli*

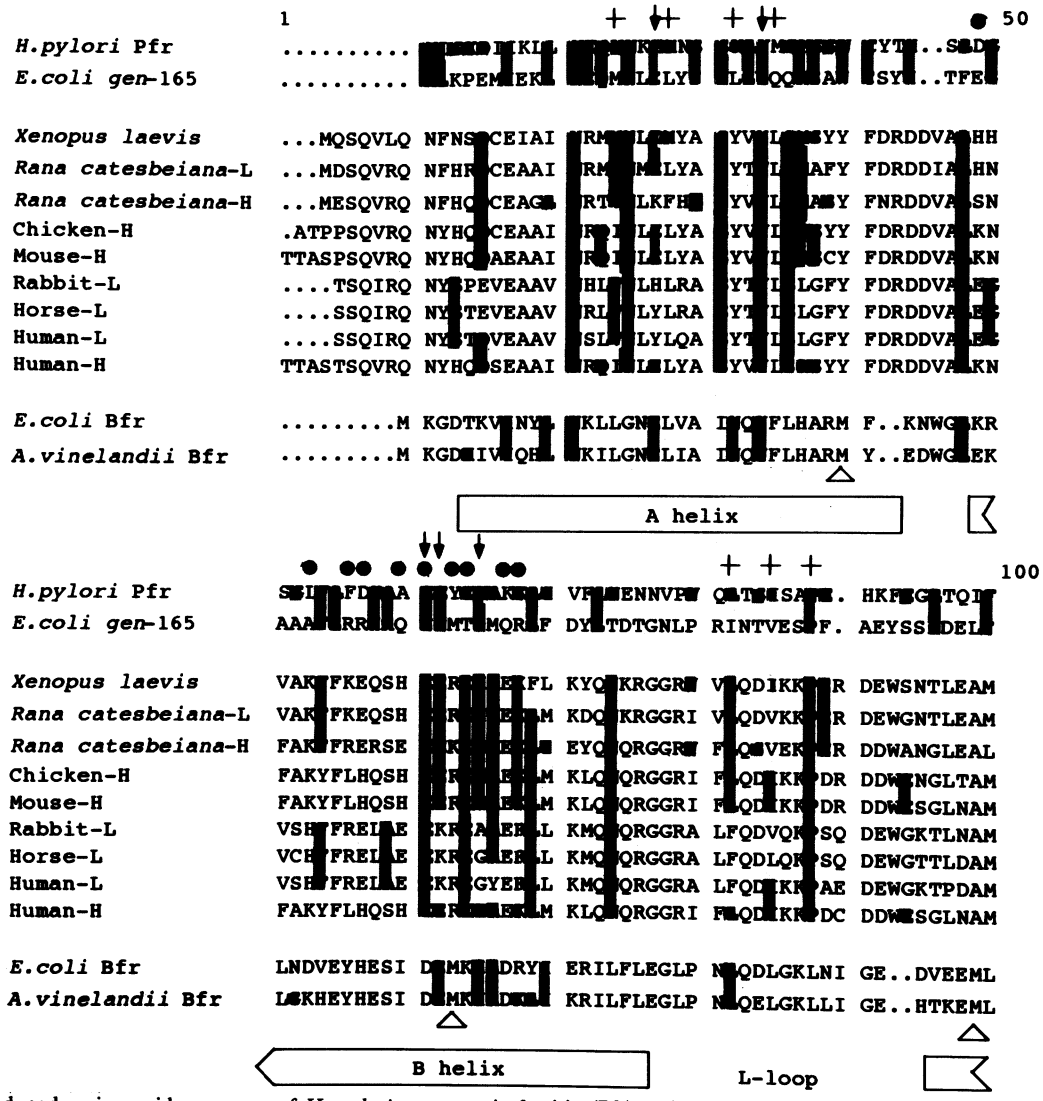
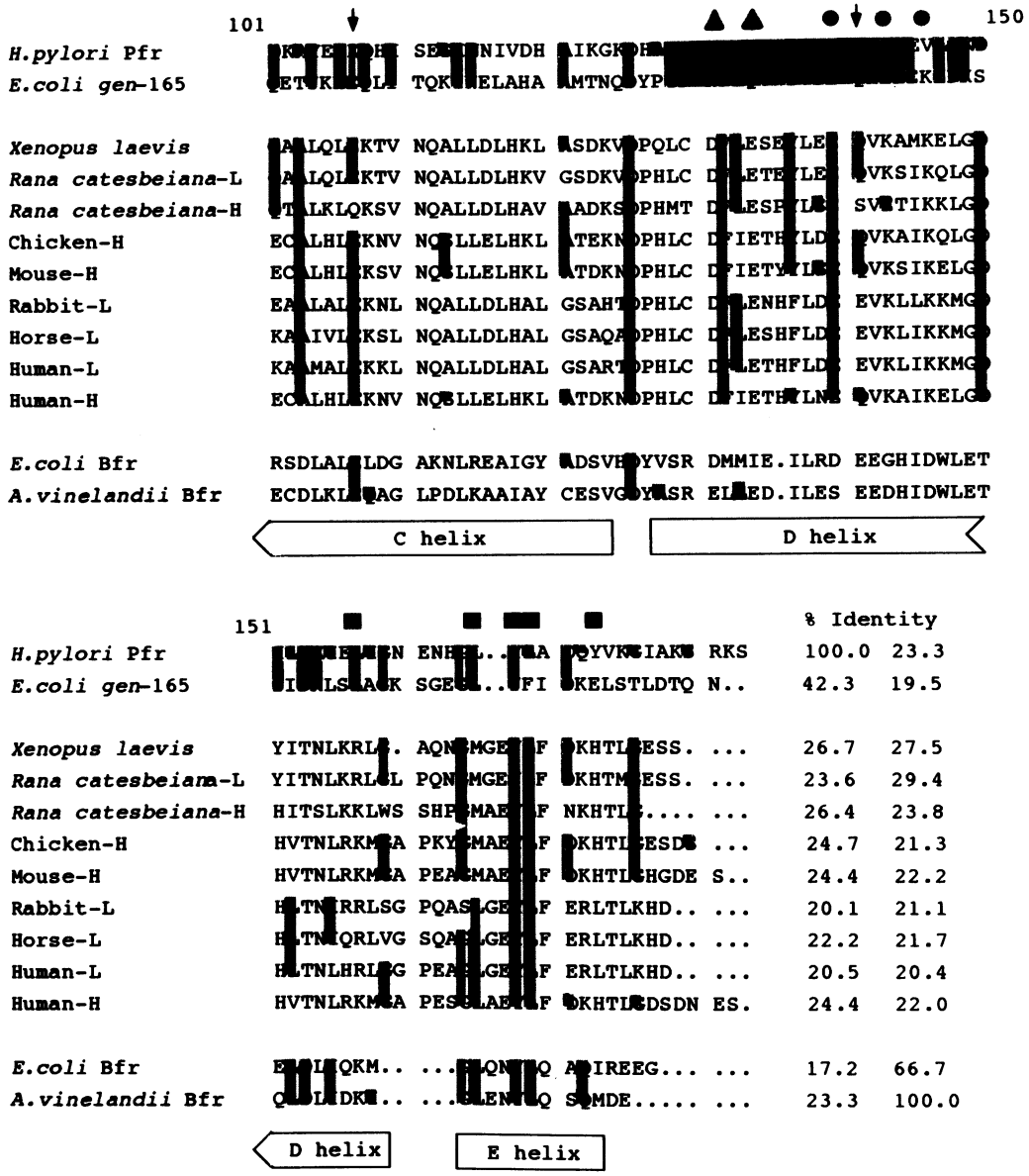


FIG. 2. Deduced amino acid sequence of *H. pylori* procaryotic ferritin (Pfr) and consensus alignments (PILEUP, Genetics Computer Group, version 7.1) for Pfr, eucaryotic ferritin, and Bfr sequences. Numbering is given for the human ferritin H chain. Amino acid residues identical to *H. pylori* Pfr are shaded. ↟, the 7 key residues forming the ferroxidase center of ferritin H chain (1, 19); △, putative heme-binding methionine residues in *E. coli* Bfr's (11); ▲, residues lining the hydrophilic channel along the threefold axis in horse spleen L-chain ferritin (7); +, hydrophobic residues at the subunit interface involving the A helix and the L loop of horse spleen L-chain ferritin (7); ●, hydrophilic residues at the inner surface of horse spleen L-chain ferritin multimers (7); ■, hydrophobic sites in horse spleen L-chain ferritin along the fourfold axis (7). Percent identity with *H. pylori* Pfr and with *A. vinelandii* Bfr is given at the lower right.

proteins also showed homology with the heme group-containing Bfr's of *E. coli* and *A. vinelandii*; the highest percentage of identity, 23.3%, was seen between the *H. pylori* 19.3-kDa protein and the Bfr of *A. vinelandii* (Fig. 2).

The *H. pylori* and *E. coli* Pfr sequences were more precisely compared with those of the eucaryotic ferritins and with those of the Bfr's of *E. coli* and *A. vinelandii* by computer-based alignment (Fig. 2). This alignment yielded four small deletions in the two Pfr's and none in the eucaryotic ferritins. Two of these deletions were localized in regions corresponding to loops in the horse L chain (7) and human H chain (17, 18, 31) (whose crystal structures have been solved); a single-amino-acid deletion localized to helix D; and a 2-amino-acid deletion localized to the region corresponding to helix E. As predicted by the Chou-Fasman and Garnier-Osguthorpe-Robson algorithms (PLOTSTRUCTURE, Genetics Computer Group version 7.1), the secondary structure of *H. pylori* Pfr also contains alpha-helical regions similar to those of the eucaryotic ferritins. In particular, both algorithms predict the regions encompassing amino acid residues 10 to 27 (based on the numbering scheme of Fig. 2), 54 to 65, 87 to 108, 113 to 119, 136 to 153, and 170 to 172 to form alpha helices.

The 7 residues of eucaryotic ferritin H chain participating in Fe chelation directly, or indirectly via water molecules, have been identified as Glu-27, Tyr-34, Glu-61, Glu-62, His-65, Glu-107, and Gln-141 (1). These critical residues forming the ferroxidase center are conserved in both the *H. pylori* and the *E. coli* Pfr's (Fig. 2). Other important residues conserved among eucaryotic ferritins are also found in the



two Pfr's, including identical or similar hydrophobic residues involving the A helix and the L loop at the intersubunit contact, a hydrophobic surface on the subunit monomer comprising one face of helix E and the Leu-158 at the C terminus of helix D, and a large number of hydrophilic residues in the B and D helices that form the inner shell of the ferritin molecule. As in eucaryotic ferritins, the two Pfr's have a high concentration of aspartate and glutamate residues in the region corresponding to the B helix (7, 28). Iron is known to protect the carboxyl groups on Glu-57 (Asp in *H. pylori*), Glu-60 (Ala in *H. pylori*), and Glu-61 (Glu in *H. pylori*) from being chemically modified (7).

Estimates of the evolutionary distance between the different ferritins (DISTANCES, Genetics Computer Group version 7.1) gave higher scores between Pfr's and eucaryotic ferritins (average, 0.38) than between Pfr's and Bfr's (average, 0.28), suggesting a shorter evolutionary distance between the first two ferritin groups. It is also possible that the eucaryotic ferritins have evolved through genetic reassortments between genes of different bacterial origin encoding Pfr's and Bfr's. In support of this view, the alignment in Fig. 2 reveals that at least one of the listed eucaryotic ferritins shares an identical residue with at least one of the four bacterial ferritins in 93 of 173 possible positions (54%).

The N-terminal region of Pfr here described is identical to the 28 reported N-terminal amino acids of a putative 19.6-kDa adhesin recently purified from *H. pylori* (5). Since Pfr is an abundant cytosolic protein (see below) like other ferritins, the presence of the protein in culture supernatant is probably due to bacterial lysis. In this context it is noteworthy that a rabbit antiserum raised against *E. coli*-produced *H. pylori* Pfr (see below) that specifically recognized Pfr in immunoblots did not inhibit *H. pylori* adhesion to human gastric mucosa in fixed tissue sections (data not shown).

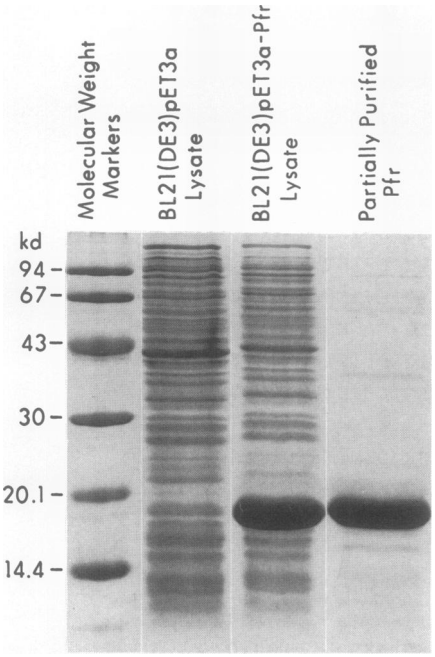


FIG. 3. Induced expression of the *H. pylori* ferritin analog in *E. coli*. Approximately 10^7 whole bacteria that had been induced with 1 mM IPTG and grown in Luria broth or the partially purified ferritin analog Pfr was analyzed by SDS–15% PAGE and visualized by Coomassie blue staining.

Pfr contains nonheme iron. To study the function of *H. pylori* Pfr, the *pfr* gene was cloned into the vector pET3a under control of the bacteriophage T7 promoter and was used in the inducible expression system described by Studier et al. (27). The representative experiment presented in Table 1 shows that a lysate of *E. coli* BL21(DE3) expressing high levels of the *H. pylori* ferritin-like protein contains almost 10-fold more iron than a lysate of control bacteria but does not contain detectable heme. This lack of additional heme may indicate that *H. pylori* Pfr does not bind iron in association with heme. This would be consistent with the amino acid sequence alignment showing that the putative heme-binding ligands Met-31, Met-52, and Met-86 (11) in *E. coli* Bfr align with a Trp, a Tyr, and an Ile residue, respectively, in *H. pylori* Pfr (Fig. 2). Comparison of the protein profile of the cell lysates of bacteria expressing the vector control, the *H. pylori* Pfr, or the partially purified Pfr demonstrates that the level of iron-containing activity parallels the abundance of the *H. pylori* protein (Fig. 3). Specifically, the protein increases from undetectable levels in vector control BL21(DE3)(pET3a) bacteria to account for up to 49% of total cellular protein (or 0.3 μg/10^6 cells) in BL21 (DE3)(pET3a-Pfr) bacteria after isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Anion-exchange chromatography increases its relative abundance by another factor of 3.

TABLE 1. Iron content of *E. coli* K-12 expressing high levels of the *H. pylori* ferritin analog

Sample	Iron (μM)[a]
PBS alone	<1.8
BL21(DE3)(pET3a) lysate	2.3
BL21(DE3)(pET3a-Pfr) lysate	21
Partially purified Pfr[b]	41

[a] The precision of the automated iron analysis method employed ranged from 2 to 10% of the measured value in this range of iron concentrations. All samples had heme concentrations of <1 μM, i.e., below the lower limit of detection of the method employed.
[b] Fractionated by FPLC as described in the text.

The Pfr protein forms paracrystalline inclusions. Both *H. pylori* P466 and *E. coli* BL21(DE3)(pET3a-Pfr) contained inclusion bodies of paracrystalline arrays. These arrays occupied a major portion of the cytoplasm in *E. coli* overproducing Pfr. Pfr-specific antibodies consistently labelled these crystalline inclusions in both *H. pylori* P466 and *E. coli* (Fig. 4), indicating that they were enriched for the Pfr protein. In the inclusions, the unit cell was tetragonal and exhibited an 8-nm periodicity.

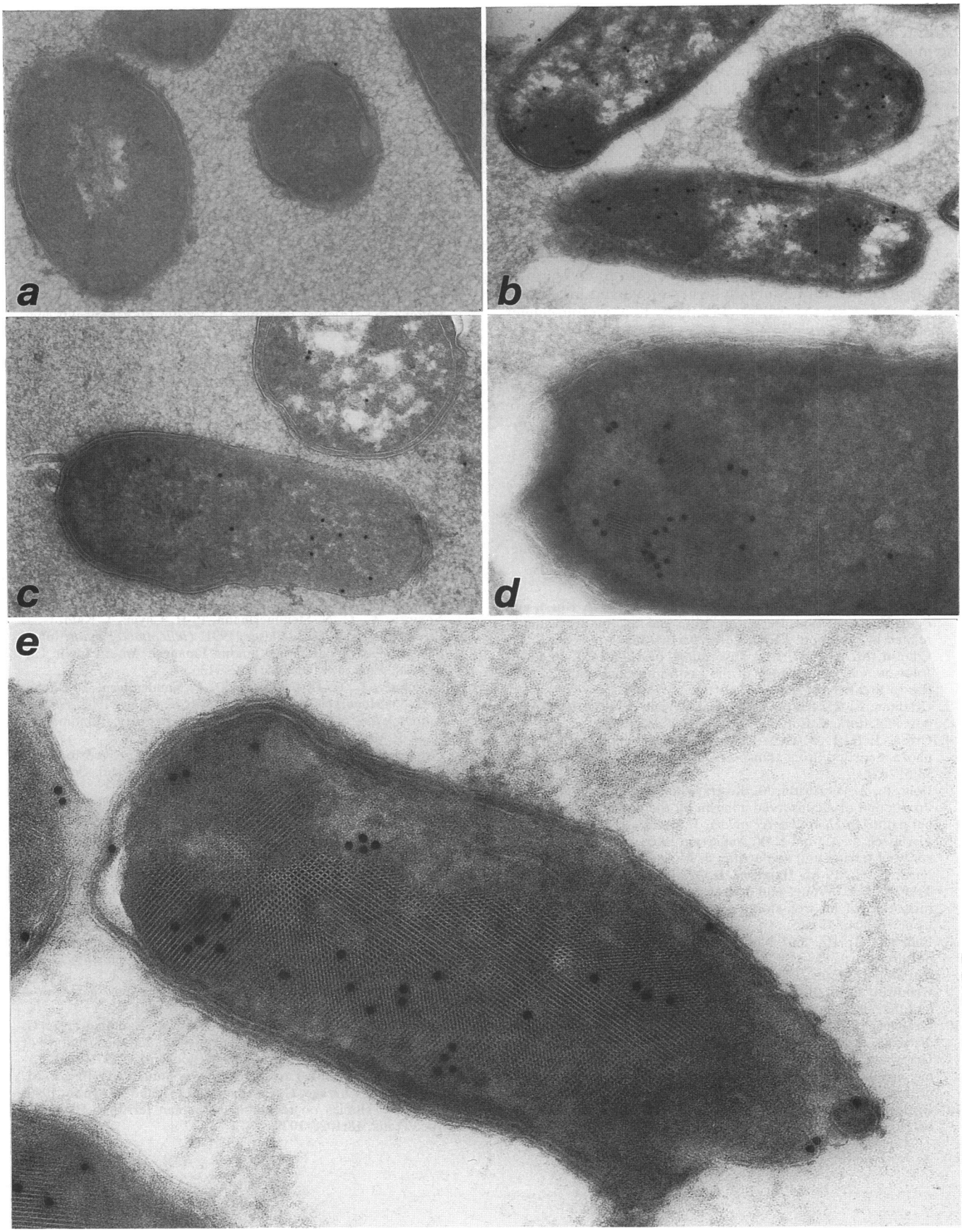
It is interesting to note that while *H. pylori* MO19 showed cytoplasmic labelling with Pfr-specific antibodies, there was no evidence of paracrystalline array formation (Fig. 4). This phenotypic difference between strains P466 and MO19 may represent different Pfr expression levels and may signal heterogeneity in Pfr metabolism in *H. pylori*.

DISCUSSION

The association of nonheme iron with Pfr is interesting to consider in conjunction with the fact that, because of the low pH in gastric juice, inorganic iron of both the ferric [Fe(III)] and ferrous [Fe(II)] forms is soluble and available for chelation and uptake in cells (2). In contrast, heme is soluble only at pHs above 6, and consequently heme-complexed iron is unlikely to be available for utilization as an iron source in the stomach (2). Although *H. pylori* may not live in the extremely low pH of gastric juice, because it exists in close association with the gastric mucus where the pH is likely higher, it is nonetheless possible that *H. pylori* in its specific habitat is not faced with iron limitation but rather with the risk of iron toxicity.

The lysate iron levels indicate that in induced *E. coli* BL21(DE3)(pET3a-Pfr) bacteria the ratio of iron to Pfr is approximately 1:1 on a molar basis (data not shown). While this ratio suggests that Pfr monomers may bind iron at the conserved ferroxidase center, it does not exclude an interaction between the subunits to form a multimeric shell around an iron core. If the Pfr protein is assembled into multimeric complexes of 24 subunits as eucaryotic ferritins,

FIG. 4. Cytoplasmic localization of Pfr by immunoelectron microscopy (25). (a) *E. coli* BL21(DE3)(pET3a) shows no labelling with the anti-Pfr antibody. Magnification, ×40,000. (b) Induced *E. coli* BL21(DE3)(pET3a-Pfr) probed with the same antibody. The antibody labels an abundant protein in the cytoplasm of the bacteria that forms dense inclusion bodies. The regular periodicity of these inclusion bodies is demonstrated in the higher-magnification view of panel e. Magnification, ×33,000. (c) *H. pylori* MO19 labelled with anti-Pfr antibody indicates that the protein is present in the cytoplasm of *H. pylori*. Magnification, ×43,000. (d) *H. pylori* P466 labelled with anti-Pfr antibody shows regular cytoplasmic paracrystalline arrays of the protein, although these arrays are not as abundant or as large as those observed in *E. coli* BL21(DE3)(pET3a-Pfr). Magnification, ×82,000. (e) A higher-magnification micrograph of induced *E. coli* BL21(DE3)(pET3a-Pfr) showing the structure of the paracrystalline arrays generated by overexpression of Pfr in *E. coli*. These arrays have a periodicity of 8 nm. Magnification, ×113,000.


a
b
c
d
e

the crystalline arrays evident by electron microscopy may conceivably be caused by a regular packing of such symmetric multimers, each with an iron center. Intracellular bacterial crystalline inclusions have so far been seen only in *Bacillus thuringiensis* expressing insecticidal crystal proteins (14). However, these crystal proteins have no known bacterial function. In the case of *H. pylori* ferritin, arrangement in paracrystalline inclusions may serve to sequester intracellular iron in a specific compartment, reducing the risk of intracellular iron-mediated toxicity.

Our results imply that *E. coli* overproducing cytosolic *H. pylori* Pfr accumulates considerably more inorganic iron than wild-type *E. coli* cells. Of interest, production of *H. pylori* Pfr in *E. coli* resulted in a significantly larger colony size on Luria-Bertani agar as compared with *E. coli* harboring the vector control. Similarly, when grown in Luria broth, *E. coli* expressing the *H. pylori* Pfr grew to a twofold-higher density in stationary phase than vector control bacteria (data not shown). It is possible that the overexpressed ferritin-stored iron in *E. coli* is utilized for cells approaching stationary phase. Additional experiments to clarify the mechanism of Pfr function in *H. pylori* and *E. coli* are in progress, as are experiments to explore possible relationships between *pfr*- and *fur*-controlled (3) iron uptake systems.

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