A Novel Non-heme Iron-binding Ferritin Related to the DNA-binding Proteins of the Dps Family in *Listeria innocua* *

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A multimeric protein that behaves functionally as an authentic ferritin has been isolated from the Gram-positive bacterium *Listeria innocua*. The purified protein has a molecular mass of about 240,000 Da and is composed of a single type of subunit (18,000 Da). *L. innocua* ferritin is able to oxidize and sequester about 500 iron atoms inside the protein cage. The primary structure reveals a high similarity to the DNA-binding proteins designated Dps. Among the known ferritins, the most similar sequences are those of mammalian L chains that appear to share with *L. innocua* ferritin the negatively charged amino acids corresponding to the iron nucleation site. In *L. innocua* ferritin, an additional aspartyl residue may provide a strong complexing capacity that renders the iron oxidation and incorporation processes extremely efficient. This study provides the first experimental evidence for the existence of a non-heme bacterial ferritin that is related to Dps proteins, a finding that lends support to the recent suggestion of a common evolutionary origin of these two protein families.

The physiological requirement of iron in a nontoxic and available form is met in all living cells by ferritins, the iron storage proteins, which are tailored to accomodate large amounts of readily mobilizable iron inside the apoferritin shell. In eukaryotes, the apoferritin coat is made of 24 subunits of two different types called H and L (21,000 and 19,500 Da, respectively) that have 55% amino acid sequence identity (1, 2). The H and L subunits are interchangeable in the assembled molecules. They share the same tertiary conformation consisting of a bundle of four antiparallel helices (A-D), a short helix (E), and a loop that connects helices B and C (3, 4). Functionally, the H subunit is characterized by the presence of a specific site for iron oxidation in the four-helix bundle, the so-called ferroxidase center, whereas the L chain contains efficient nucleation sites that face the protein cavity and are thus able to favor iron accumulation (5, 6).

In bacteria, notably in Gram-negative ones, the ferritin-like proteins shown functionally to bind inorganic iron can be classified in two different categories: the heme-b containing “bacterioferritins” (Bfr) found in microorganisms such as *Escherichia coli* (7, 8) and *Azotobacter vinelandii* (9) and non-heme containing ferritins such as those expressed by *Helicobacter pylori* (10, 11) and by the *E. coli* gen-165 gene (12, 13). Bacterioferritins are the most studied ones. They are formed by a single subunit that has a low sequence similarity (about 20%) with eukaryotic ferritins but that is characterized by the same four-helix bundle conformation and by the presence of the seven amino acid residues that constitute the ferroxidase center of the H-type chains (5). The non-heme containing ferritin-like proteins expressed by *H. pylori* and the *E. coli* gen-165 gene have only about 30% sequence similarity with bacterioferritins, but sequence alignment and modelling studies predict the conservation of the ferroxidase center residues (14). Consistent with this prediction, the *H. pylori* protein has been shown to bind inorganic iron (10, 11).

Recently, Evans et al. (15) identified four new putative prokaryotic ferritins using gene sequence analysis and an alignment that introduces a number of gaps in regions of the protein structure that correspond to α-helices. On this basis, the neutrophil-activating protein A encoded by the *H. pylori* NapA gene (15), the protein encoded by the metal-regulated *MrgA* gene of *Bacillus subtilis* (16, 17), and proteins of unknown function in *Treponema pallidum* (18) and *Anabaena variabilis* display sequence similarity with mammalian and proven bacterial ferritins and conserve all or most of the seven ferroxidase center amino acids. Evans et al. (15) also found a striking similarity between this new group of putative ferritins and the DNA-binding protein from starved cells of *E. coli* (20), which belongs to the Dps family, a diverse group of bacterial, stress-induced polypeptides that bind DNA. Almost at the same time, Peña and Bullerjahn (21), in a study of the DpsA protein of *Synechococcus sp.*, assigned all of the putative ferritins of Evans et al. (15) to the Dps family also in view of the demonstration that the *B. subtilis* MrgA protein binds DNA (17). Unlike most members of the Dps family, the *Synechococcus* DpsA protein contains heme, a peculiarity that was ascribed to the high similarity (>60%) between its C-terminal domain and the C-terminal half of heme-containing bacterioferritins (21). On the basis of these considerations, Peña and Bullerjahn proposed a common evolutionary origin for the Dps and bacterioferritin/ferritin superfamilies and speculated that the Dps proteins may have evolved as heme- or metal-binding proteins that later acquired a DNA-binding activity.

This intriguing situation and the lack of biochemical data on...
Dps-related Ferritin from *L. innocua*

ferritins extracted from Gram-positive bacteria suggested that the metal-binding ancestor of the Dps proteins hypothesized by Peña and Bullerjahn (21) could perhaps be found among such bacteria. *Listeria innocua* was chosen as the potential source of ferritin, also in view of the importance of iron in the infection process caused in humans and animals by the pathogenic species, *Listeria monocytogenes* (22, 23). A multimeric protein able to incorporate inorganic iron, and hence functioning as an authentic ferritin, was purified and characterized. *L. innocua* ferritin, like all bacterial ferritins, is formed by one type of subunit (18,000 Da) whose amino acid sequence shows a high similarity to the Dps proteins and does not appear to contain the ferroxidase center residues characteristic of eukaryotic ferritins. A further unique property of *L. innocua* ferritin is the molecular mass of the native polymer (240,000 Da), which is suggestive of a different mode of subunit assembly with respect to tetracoseramic (24-mer) ferritins, which are all characterized by a mass of 450,000–500,000 Da.

**MATERIALS AND METHODS**

Enzymes and chemicals were purchased from the following suppliers. Trypsin (code TRTPCK) was from Worthington, pepsin was fromSigma, and ammonium bicarbonate was from Merck, Darmstadt, the liquid chromatography solvents (HPLC grade) were from Carlo Erba Reagenti, and sequence-grade chemicals were from Perkin-Elmer.

**Bacterial Strain and Growth Conditions—** *L. innocua* was grown routinely in brain-heart infusion (BHI) medium (Merek). A preculture of 500 ml of *L. innocua*, obtained after an incubation period of 14 h in BHI medium (Fe(III) 20 ppm), was used as inoculum of a 50 liter fermentation vessel. After 15 h of growth, under agitation and aeration at 37 °C, the culture was centrifuged, and 110 g (wet weight) of cells was obtained.

**Ferritin Purification and Characterization—** A 110-g sample of packed cells was suspended in 360 ml of 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and disrupted in a French press. Debris was removed by centrifugation (15,000 rpm for 40 min), the supernatant was heated at 70 °C for 10 min and rapidly cooled. After centrifugation (15,000 rpm for 30 min), this material was treated with ammonium sulfate (80% w/v); the precipitate was collected by centrifugation (10,000 rpm for 20 min), dissolved in 200 ml of 20 mM Tris-HCl, pH 7.4, and dialyzed against the same buffer. To remove DNA, the material was treated with a solution of streptomycin sulfate (1% w/v) for 30 min at room temperature and centrifuged (15,000 rpm for 15 min). The protein was further purified by fast protein liquid chromatography (FPLC) using a Mono-Q column (Pharmacia Biotech Inc.) equilibrated with 20 mM Tris-HCl, pH 7.4, and eluted with a linear gradient of 0–1.5 M NaCl in the same buffer. Ferritin eluted at 0.25 M NaCl. In some preparations, gel filtration chromatography on an FPLC-G200 Sephadex column (Pharmacia) was carried out as a further purification step. The purity achieved during the various steps of the procedure was checked by SDS-polyacrylamide gel electrophoresis according to Laemmli (24) and by non-denaturing gel electrophoresis (25). Denaturing gels were stained with Coomassie Blue, and native gels were also stained for iron with Peri- nBlue. The data presented refer to five different protein preparations. The yield of pure *L. innocua* ferritin was unaffected by omitting the heating step during the purification procedure.

Protein concentration was determined by the method of Lowry et al. (26) or by using the extinction coefficient E (1 cm, 1 mg/ml) = 1.2 at 280 nm that was calculated from the absorption spectrum and the protein content determined by amino acid analysis. The iron content of the native protein was determined as the 2–2’-bipyridyl complex at 520 nm (27). Iron was removed from ferritin by incubation for 24 h in 0.3% sodium dithionite in 50 mM MES-NaOH, at pH 6.0, containing 2–2’-bipyridyl added to chelate sparingous ferrous iron.

Iron incorporation experiments were performed using Fe(NH₄)₂(SO₄)₂ as iron donor. All iron solutions were prepared in Thunberg tubes, kept anerobically under a nitrogen atmosphere, and used within a few hours from their preparation. The iron oxidation and incorporation kinetics were followed spectrophotometrically at 310 nm upon addition of different amounts of iron to apoferritin solutions equilibrated in air. The extinction coefficient of micellar iron at 310 nm was taken as 450 (1%, 1 cm), the value used for horse spleen ferritin (28). For kinetic control, the reaction of the ferritin was monitored in parallel. After iron incorporation, the samples were subjected to gel electrophoresis under non-denaturing conditions on 6% polyacrylamide gels. The gels were stained for iron with potassium ferrocyanide and for protein with Coomassie Blue.

Fluorescence measurements were performed using a SPEX 2000 single photon counting spectrometer by excitation of the aromatic amino acid residues at 280 nm at 20 °C in 20 mM Tris-HCl, pH 7.4. The bandwidths of the excitation and emission monochromators were 2 nm. Circular dichroism spectra were measured with a Jeol J-710 spectropolarimeter in the near and far UV regions were 20 °C in 20 mM Tris-HCl, pH 7.4. The molar ellipticity (degree cm² mol⁻¹) in the far UV region ([θ]m) was calculated on the basis of a mean residue molecular weight of 116 and in the near UV region ([θ]m) on the basis of the polypeptide chain molecular mass (18,000 Da).

Sedimentation velocity experiments were carried out on a Beckman Instruments Optima XL-A analytical ultracentrifuge at 30,000 rpm and 10 or 20 °C over the concentration range 0.2–0.5 mg/ml. The gradient of protein concentration in the cells was determined by absorption scans along the centrifugation radius at 280 and 510 nm with a step resolution of 50 mm. Sedimentation coefficients were evaluated with the software provided by Beckman and were reduced to s₂₀w by standard procedures. The data have been transformed to a plot of (g(s) versus s²), an unnormalized differential distribution of apparent sedimentation coefficients, that is geometrically similar to the more commonly used Schlieren plot (dcdv versus r).

The sedimentation equilibrium experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge at 5,000 and 10,000 rpm and 10 °C over the concentration range of 0.2–0.5 mg/ml. The data (step resolution 0.001 cm, 20 averages/scan) were analyzed with the Ideal 1 software provided by Beckman.

For electron microscopy experiments, the samples were negatively stained. One drop of *L. innocua* ferritin obtained by incubation with 500 iron atoms/apoferritin molecule in MOPS-NaOH, pH 7.0, was placed on a carbon film mounted on a 300-mesh copper grid, immediately moved, and stained with 2% phosphotungstic acid (pH 7.2). Micrographs were taken at ×50,000 with a Zeiss EM 902 electron microscope operating at 60 kV. The measurement of the diameter of the particles was performed, on about 100 particles, by a graduated lens on micrographs with a final magnification of ×300,000.

Amino Acid Sequence Analysis—The purified protein (10 μg) was subjected to vapor phase hydrolysis in 6 N HCl at 110 °C for 24 h. Amino acid analysis was performed with a Beckman System Gold analyzer equipped with an ion-exchange column and post-column derivatization with ninhydrin. Although the amino acid analysis revealed the absence of cysteines, the protein sample (1.7 mg) was suspended in 0.5 ml of 0.5 M Tris-HCl pH 7.5 (containing 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 12 μM dithiothreitol), and incubated for 3 h at 55 °C. Then, 4-vinlypyridin (90 μmol/10 μl) was added, and after a 10-min incubation, the protein was desalted by HPLC using a guard cartridge (C8, 4.6 × 30 mm). This treatment was necessary in order to denature the protein, which is otherwise resistant to proteolytic attack. An aliquot (0.7 mg) of the denatured protein was suspended in 0.5 ml of 0.1 M ammonium bicarbonate and incubated at 37 °C for 3 h after addition of 14 μg of trypsin. A second aliquot of protein (0.5 mg) was dissolved in 0.2 ml of 5% (v/v) formic acid and incubated with 10 μg of pepsin at 25 °C for 5 min. The peptide mixtures obtained following enzymatic digestions were purified immediately after incubation with proteases and without lyophilization. The last aliquot of protein was dissolved in 0.2 ml 70% (v/v) formic acid, incubated with 5 mg CNBr for 24 h at room temperature, in the dark, and lyophilized.

The peptide mixtures were purified by HPLC using a Beckman System Gold chromatographer on a macroporous reverse-phase column (Agappece RP-300, 4.6 × 250 mm, 7 μm, Brownlee Labs) eluted with a linear gradient from 0 to 35% acetonitrile in 0.2% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml/min. Elution of the peptides was monitored using a diode array detector and a UV light at 220 and 280 nm.

The amino acid sequence of peptide samples was determined by automated Edman degradation using an Applied Biosystems model 476A sequencer. Samples (0.1–0.5 nmol) were loaded onto polyvinylidenem difluoride membranes (ProBlott, Applied Biosystems), coated with 2 μl polybrene (100 mg/ml of 50% methanol) and run with a Blott cartridge using an optimized gas-phase fast program. N-terminal se
sequence analysis of the protein was performed on samples (10 μg) electrotransferred on ProBlott membranes after SDS-polyacrylamide gel electrophoresis (29) using a liquid-phase fast program.

Peptides were numbered retrospectively according to their location in the sequence, starting from the N terminus. Tryptic peptides were designated with T, peptic peptides with P, and CNBr peptides with B.

**Structure Comparison—** A search of the SwissProt Data Base, pairwise and multiple sequence alignments, as well as prediction of secondary structures were carried out with the programs FASTA, GAP, PILEUP, and PEPTIDESTRUCTURE, respectively, from the Genetic Computer Group sequence analysis software package (GCG, Version 8).

**RESULTS**

Production and Purification of Ferritin from *L. innocua*—Crude extracts of *L. innocua* cells show, in native gel electrophoresis, the presence of a band that is characterized by a significantly faster mobility relative to horse spleen ferritin and stains specifically for iron. On the basis of this finding, a purification protocol has been utilized that takes advantage of the high thermal stability of ferritins and includes a heating step at 70 °C. The subsequent purification procedure, consisting of an ion exchange chromatography and a gel filtration step, yields about 10 mg of pure protein, as judged by gel electrophoresis in denaturing conditions, from 100 g of cells. In SDS gradient pore acrylamide gel electrophoresis, *L. innocua* ferritin appears to be composed by only one type of subunit with a molecular mass of about 18,000 Da. This value is similar to that of other bacterial ferritins and is lower with respect to those of the H and L mammalian ferritin polypeptide chains.

Amino Acid Sequence Determination—The complete amino acid sequence of ferritin from *L. innocua* is reported in Fig. 1. The subunit has 156 amino acid residues, yielding a molecular mass of 18,048 Da, which corresponds to the value obtained by electrophoretic analysis. The sequence was deduced following N-terminal sequence analysis of the protein up to residue 57 and isolation and identification of an almost complete set of tryptic peptides, which were ordered with the help of overlapping peptides produced by pepsin and cyanogen bromide cleavages. The reported sequence is in good agreement with the amino acid composition of the protein. The C-terminal extremity of the protein was identified on the basis of the evidence that the same sequence, ending with a C-terminal glutamic acid, was present after complete sequencing of peptides obtained from two different types of cleavage that are known not to occur at Glu-Xaa peptide bonds (see peptides T12 and B7).

Secondary structure prediction, performed according to Garnier et al. (31) shows the presence of four α-helices in the regions corresponding to positions 9–28, 40–67, 76–119, and 124–137. This four-helix pattern is strongly reminiscent of the four-helix bundle characteristic of mammalian ferritins (3, 4).

Molecular Weight Determination and Electron Microscopy Data—In sedimentation velocity experiments, native *L. innocua* ferritin is characterized by the presence of a single symmetrical component with *s*₂₀,₀₀₀ = 11 S (Fig. 2a); this value does not change upon removal of the iron, indicating that the iron content of the native protein is very low. For a spherical molecule, this sedimentation velocity yields a molecular mass of 240,000 Da using the partial specific volume calculated from the amino acid composition (V = 0.734) and the nomogram of Wyman and Ingalls (32).

In order to obtain a direct determination of the molecular mass of the apoprotein, sedimentation equilibrium experiments were carried out. The data analysis yields 240,000 ± 1,400 Da, a value which has to be compared with 450,000–500,000 Da typical of mammalian ferritins (1, 3).

Negative staining electron microscopy shows that *L. innocua* ferritin is a spherical protein shell that encloses an electron-dense core just like horse spleen ferritin (Fig. 3). However, the size of the two molecules differs significantly. The dimensional analysis yields an average diameter of 12.15 nm for the horse spleen ferritin molecules and of 10.14 nm for the *L. innocua* ferritin ones. The corresponding volumes, assuming a spherical shape, are about 940 and 545 nm³, respectively.

Iron Incorporation Experiment—The iron content of the native protein is low and corresponds to 5–10 atoms/molecule (240,000 Da). The iron-free protein was obtained by reduction with sodium dithionite at low concentration (0.3% w/v).

Native *L. innocua* ferritin and the iron-free protein are able to oxidize and incorporate iron as indicated by electrophoretic analysis of the products obtained upon incubation with different amounts of ferrous iron in the presence of molecular oxygen. A progress curve of iron uptake by *L. innocua* apoferritin upon addition of 500 Fe/molecule is shown in Fig. 4; the half-time corresponds to 90 s and is similar to that of human rH...
apoferritin analyzed in parallel. Under these experimental conditions, all the iron added appears to be incorporated by *L. innocua* apoferritin. Upon addition of \( \approx 1,000 \) Fe/molecule precipitation of ferric iron outside the protein is observed.

Further evidence that iron is incorporated by *L. innocua* ferritin is provided by sedimentation velocity analysis of samples loaded with iron at pH 7.0. The sedimentation patterns show the presence of a single homogeneous peak characterized by a sedimentation coefficient, \( s_{20,W} = 21–22 \) S (Fig. 2b), which is significantly higher than that characterizing the iron-free protein (\( s_{20,W} = 11 \) S). The protein loaded with iron at pH 6.5 displays a fast sedimenting component of similar sedimentation coefficient (\( s_{20,W} = 21–22 \) S) although about 10% apoferritin is still present (Fig. 2c).

**Spectroscopic Characterization**—The absorption spectrum of *L. innocua* apoferritin in the ultraviolet region is characterized by a broad peak centered at 279 nm with a shoulder at 292 nm attributable to tryptophan residues, whereas the shoulders at 264 and 271 nm are attributable to tyrosines. The shoulder at 287 nm and the well-resolved negative peak at 294 nm can be ascribed to the 0 + 850 cm\(^{-1}\) \( ^{13} \)N and the 0 - 0 cm\(^{-1}\) \( ^{15} \)N transitions of tryptophan residues, respectively. These assignments are consistent with the broadness of the absorption band and the lack of coincidence between the absorption and CD spectra (33).

The intrinsic fluorescence spectrum is characterized by an emission maximum at 325 nm upon excitation at 280 nm (Fig. 5c). The emission wavelength is similar to that of the human rH homopolymer but occurs at higher wavelengths than in the L-rich horse spleen apoferritin. As in the case of the rH homopolymer, it can be ascribed to tryptophan residues exposed to solvent (34, 35).

**DISCUSSION**

The presence of a polymeric protein in *L. innocua*, which is able to bind and incorporate iron, is of great interest since it represents the first example of an authentic ferritin that bears a high sequence similarity with Dps proteins and thus lends support to the evolutionary link between ferritins and Dps proteins proposed by Peña and Bullerjahn (21). *L. innocua* ferritin is also the first characterized ferritin from a Gram-positive bacterium.

Native *L. innocua* ferritin contains a very small amount of iron but incorporates the metal efficiently at a rate that resembles that of the human rH homopolymer. The maximum iron binding capacity corresponds to about 500 atoms and is thus much smaller than that of the mammalian molecule, which can harbor up to 4500 iron atoms. This difference, however, is consistent with the molecular mass of the *L. innocua* polymer, which is lower than that of the mammalian one, namely 240,000 Da as compared with 450,000–500,000 Da. It is also consistent with the electron microscopy data of Fig. 3 which show that, in *L. innocua* ferritin, the protein cage and the iron core are both smaller than in horse spleen ferritin. A distinctive feature of reconstituted *L. innocua* ferritin is its homogeneity in iron content, a characteristic that may be related to the structural basis of the iron oxidation and incorporation processes. Thus, the iron cores obtained at pH 7.4 and 6.5 are all very similar and contain about 500 iron atoms, whereas in horse spleen ferritin, the reconstituted iron cores vary greatly in size (36).

Having established that the 240,000 Da iron-binding protein isolated from *L. innocua* behaves functionally as a ferritin, the first question that arises concerns its relationship with the different classes of the ferritin/bacterioferritin superfamily.

However, searching in the SwissProt Data Base with the *L. innocua* ferritin sequence as probe did not retrieve any of the proven ferritin sequences but retrieved with the highest score eight protein sequences all belonging to the Dps family. The alignment of these sequences, obtained using the program PILEUP, is reported in Fig. 6. The comparison shows that identical amino acid residues are clustered in the region corresponding to positions 36–73 (the numbering refers to the alignment reported in the figure). The residues marked with aster-
ferritin sequence was then compared with the different classes of proven ferritins, i.e. heme and non-heme containing bacterial ferritins and mammalian ferritins. As discussed by Andrews et al. (14) and Grossman et al. (37), the sequences within each class (Fig. 7) have a high degree of identity, whereas the percentage identity among the three different classes is low. However, the residues that constitute the ferroxidase center (indicated by asterisks in Fig. 7) are conserved in all the sequences except in the L subunits of mammalian ferritins, which lack the ferroxidase activity (5).

Attempts to align the L. innocua ferritin sequence with each of the sequences given in Fig. 7 failed to show significant similarities. In particular, there was no evidence for the conservation of all or part of the canonical ferroxidase center residues.

However, for the L mammalian ferritin chains, a stretch of five identical residues (A/YERLL, positions 66–70 in Fig. 7) was observed. Upon alignment of this stretch, additional conserved residues spread all over the sequence became apparent (boxed residues in Fig. 7). The negatively charged amino acids at positions 49, 57, 60, 64, and 67 (corresponding to positions 44, 52, 55, 59, and 62 of the L. innocua ferritin sequence, see Fig. 1) located in the B helix of the four-helix bundle are of special interest for their possible functional implication. In mammalian L chains, all these residues protrude from the B helix into the internal cavity of the assembled molecule. Those at positions corresponding to 57 and 60 are the main sites of iron core nucleation on the basis of site-directed mutagenesis experiments although carboxylates at positions 49, 64, and 67 have been suggested to participate in the process as well (38). In L. innocua ferritin, a helical wheel projection (Fig. 8) reveals that an additional negative charge, carried by Asp-53 (position 48 of the L. innocua ferritin sequence), is present between glutamic acids 57 and 60. However, it does not appear to be able to enhance the rate of Fe(II) oxidation by atmospheric oxygen as shown by the behavior of low molecular weight Fe(III)-complexing agents (like EDTA, nitrilotriacetic, and citrate). These compounds at neutral pH values promote oxidation of Fe(II) at rates similar to those observed for L. innocua ferritin (39). In L. innocua ferritin, therefore, Fe(II) oxidation and Fe(III) nucleation would occur within the same cluster of negatively charged residues facing the internal cavity of the molecule, a hypothesis that is consistent with the homogeneity of the iron cores produced in the reconstituted protein.

The helical wheel projection of Fig. 8 also permits other considerations that concern the relationship between ferritins and Dps proteins and are based on the assumption just used, namely that the B helix has the same orientation in the L. innocua and mammalian ferritin polymers. The amino acid residues common to the B helix of the four-helix bundle are of special interest for their possible functional implication. In particular, there was no evidence for the conservation of all or part of the canonical ferroxidase center residues (indicated by asterisks in Fig. 7). The negatively charged amino acids at positions 49, 57, 60, 64, and 67 (corresponding to positions 44, 52, 55, 59, and 62 of the L. innocua ferritin sequence) are conserved in all the sequences except in the L subunits of mammalian ferritins, which lack the ferroxidase activity (5).

Table I

| Fer_Lisin | Mrga_Bacsu | Napa_Helpy | Yd49_Haerin | Tpf1_Trepa | Dps_Ecoli | Ylt2_Anava | DpsA_Syene |
|-----------|------------|------------|-------------|------------|-----------|------------|------------|
| Fer_Lisin | 36.9       | 33.3       | 24.2        | 28.6       | 28.4      | 22.7       | 22.9       |
| Mrga_Bacsu| 33.3       | 31.5       | 27.4        | 20.0       | 25.5      | 22.9       | 22.9       |
| Napa_Helpy| 24.2       | 27.4        | 22.7        | 20.0       | 25.5      | 22.9       | 22.9       |
| Yd49_Haerin| 28.6       | 29.4        | 23.6        | 26.6       | 27.5      | 22.6       | 22.6       |
| Tpf1_Trepa | 30.2       | 29.4        | 23.6        | 26.6       | 27.5      | 22.6       | 22.6       |
| Dps_Ecoli | 28.6       | 20.0        | 25.5        | 18.9       | 27.5      | 25.1       | 21.3       |
| Ylt2_Anava| 28.4       | 27.4        | 22.7        | 20.0       | 25.5      | 22.6       | 22.6       |
| DpsA_Syene| 24.3       | 25.8        | 23.6        | 26.6       | 25.1      | 31.9       | 23.4       |
| % sequence identity |            |            |             |            |           |             |            |

Fig. 6. Amino acid sequence comparison among the protein sequences retrieved from the SwissProt Data Base using L. innocua ferritin as probe. The alignment was obtained using PILEUP. Fer_Lisin, ferritin from L. innocua; Dps_Ecoli, DNA-binding protein from streptococcal cells of E. coli (20); DpsA_Syene, DNA-binding hemoprotein from S. synchrochoccus sp. strain PCC7942 (21); Dps_Heldu, Dps protein from H. enteritidis, Dps protein from B. subtilis (20); Dps_Bacill, ferritin from B. subtilis (19). The percentage identity among the sequences given in Fig. 7 failed to show significant similarities. In particular, there was no evidence for the conservation of all or part of the canonical ferroxidase center residues.
The characterization of *L. innocua* ferritin, which behaves as an authentic ferritin endowed with iron oxidation/incorporation properties but whose sequence is related to those of the Dps proteins, has provided an unexpected proof for the proposed evolutionary relationship between the ferritin/bacterioferritin and the Dps superfamilies and calls for further biochemical studies on representative members of the two groups of proteins.

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