BioMetals **17:** 197–202, 2004.

Iron and proteins for iron storage and detoxification

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Key words: Dps proteins (DNA-binding proteins from starved cells), ferritin, iron storage-detoxification

Abstract

Iron is required by most organisms, but is potentially toxic due to the low solubility of the stable oxidation state, Fe(III), and to the tendency to potentiate the production of reactive oxygen species, ROS. The reactivity of iron is counteracted by bacteria with the same strategies employed by the host, namely by sequestering the metal into ferritin, the ubiquitous iron storage protein. Ferritins are highly conserved, hollow spheres constructed from 24 subunits that are endowed with ferroxidase activity and can harbour up to 4500 iron atoms as oxy-hydroxide micelles. The release of the metal upon reduction can alter the microorganism-host iron balance and hence permit bacteria to overcome iron limitation. In bacteria, the relevance of the Dps (**D**NA-binding **p**roteins from **s**tarved cells) family in iron storage-detoxification has been recognized recently. The seminal studies on the protein from *Listeria innocua* demonstrated that Dps proteins have ferritin-like activity and most importantly have the capacity to attenuate the production of ROS. This latter function allows bacterial pathogens that lack catalase, e.g. *Porphyromonas gingivalis*, to survive in an aerobic environment and resist to peroxide stress.

Iron is essential for most organisms since it serves as cofactor in several enzymes and as catalyst in electron transport processes, yet it is poorly available and potentially toxic. Thus, Fe(III), the stable oxidation state of the metal at neutral pH values, forms insoluble hydroxy-aquo complexes that are in equilibrium with a free Fe(III) concentration around 10^{-18} M, whereas bacteria, for example, need iron concentrations around 10^{-7} M for growth. Further, free iron in the presence of oxygen is a dangerous metal for the cell as it potentiates the non-enzymatic production of reactive oxygen species (ROS) through the following reactions:

$$\mathrm{Fe(II)} + \mathrm{O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{O_2^-} \quad (1)$$

$$2\mathrm{O_2^-} + 2\mathrm{H^+} \rightarrow \mathrm{H_2O_2} + \mathrm{O_2} \quad (2)$$

$$\mathrm{H_2O_2} + \mathrm{Fe(II)} \rightarrow {}^{\bullet}\mathrm{OH} + \mathrm{OH^-} + \mathrm{Fe(III)}. \quad (3)$$

Hydroxyl radicals in particular can damage a number of cellular components, like DNA, proteins and membrane lipids.

All organisms therefore have developed strategies that allow them to acquire iron, and to solubilize and store it in a non toxic, readily available form. Bacteria use a variety of iron acquisition systems to bridge the enormous gap between iron supply and demand. Gram-negative bacteria are characterized by numerous iron-uptake mechanisms that comprise the utilization of iron sources presented by the host (e.g. iron-loaded lactoferrin or transferrin, heme, hemoglobin), the secretion of siderophores characterized by a very high affinity and specificity for Fe(III), and the synthesis of iron-transport systems that deliver the metal into the bacterial cell via specific receptors (Cornelissen & Sparling 1994, Schryvers & Stojilijkovic 1999, Wandersman & Stojiljkovic 2000). Gram-positive bacteria seem to contain relatively few iron-uptake mechanisms. Specific to Gram-positive bacteria is the reduction of Fe(III) with ferric reductases located on the bacterial surface that permits the direct import of Fe(II) into the cell (Johnson *et al.* 1991, Deneer *et al.* 1995)

The host and bacteria use the same general strategies to counteract the reactivity of iron. Typically, intracellular iron is sequestered reversibly in ferritins and ROS are detoxified by means of specific enzymes (e.g. superoxide dismutases and catalase that act respectively on the superoxide anion and on hydrogen peroxide). Recently however, a protein family distinct from ferritins has been recognized to possess iron storage and detoxification capacity in bacteria. This is the Dps family (**D**NA-binding **p**roteins from **s**tarved cells) named after the prototype expressed in *Escherichia coli* under starvation or oxidative stress (Almiron *et al.* 1992).

Ferritin has a highly conserved molecular architecture in all organisms (Harrison & Arosio 1996). The protein moiety, apoferritin, is an extremely stable hollow sphere that can harbour up to 4500 iron atoms as ferric oxy-hydroxide micelles. Apoferritin is constructed from 24 identical or very similar subunits that are folded in a four-helix bundle and contain a ferroxidase site, located within the four-helix bundle of individual subunits, and micelle nucleation sites facing the internal cavity. The subunits are assembled with 432 symmetry such that small hydrophobic and hydrophilic channels are formed at the 4-fold and 3-fold symmetry axes, respectively. These channels permit passage of iron and other ions or small molecules. Iron in particular is guided to the 3-fold channels by a negative electrostatic gradient, enters the channels and reaches the ferroxidase sites (Douglas & Ripoli 1998). In fact, although ferritins store iron in the Fe(III) form, the metal is bound as Fe(II) and oxidized at the ferroxidase site where the iron ligands are highly conserved throughout evolution. After oxidation, iron moves to the internal cavity of the apoferritin shell which is rich in carboxylate residues that provide the nucleation sites of the ferric oxy-hydroxide micelles. When required for intracellular metabolism, ferritin iron can be mobilized upon reduction of the oxy-hydroxide core. The nature of the physiological reducing agent is still unknown though ascorbate and flavins can act as ferritin iron mobilizing agents *in vitro* (Jones *et al.* 1978, Takagi *et al.* 1998). Importantly, the release of bacterial ferritin iron upon reduction may alter the microorganism-host balance and thus permit bacteria to overcome iron limitation.

A closer look at the ferroxidation reaction reveals that ferritins of various organisms are characterized by different chemistries of iron deposition. In mammalian ferritins, iron is oxidized pairwise by molecular oxygen with formation of a peroxo intermediate. Molecular oxygen is reduced to hydrogen peroxide which is released into the bulk solution where catalase can disproportionate it to water and molecular oxygen (Yang *et al.* 1998); in part it may be utilized by ferritin itself in a detoxifixation reaction (Zhao *et al.* 2003). In bacteria possessing 'canonical' ferritins these carry out the ferroxidation reaction just as mammalian ferritins and have an iron storage role. However, in some bacteria heme-containing ferritins, the so-called bacterioferritins, may be present in addition to the 'canonical' ones (Cheesman *et al.* 1993). The case of *E. coli* is prototypical: it possesses two 'canonical' non-heme ferritins, EcFtnA and EcFtnB, and a heme-containing bacterioferritin, EcBFR. In EcBFR, up to 12 protoporphyrin IX groups can bind between 2-fold symmetry related subunits, and are ligated by two methionine residues (Frolow *et al.* 1994). The function of the heme groups is still unknown although a role in mediating iron core reduction and iron release from the protein has been proposed (Andrews 1998). In bacterioferritins the ferroxidation chemistry has distinct features relative to the 'canonical' mammalian protein. Thus, hydrogen peroxide carries out the pairwise oxidation of Fe(II) better than oxygen; and the product of dioxygen reduction is water and not hydrogen peroxide. The consumption of hydrogen peroxide at the ferroxidase site parallels the inhibition of the odd-electron oxidation of Fe(II) by H_2O_2 (reaction 3) and hence accounts for the capacity of bacterioferritins to attenuate the production of hydroxyl radicals. In turn, this capacity implies that bacterioferritins are endowed with specific detoxifying properties towards the damaging action of ROS. It is therefore likely that bacterioferritin serves to protect cellular components from oxidative damage and hence plays a role in oxidative stress relief (Bou-Abdallah *et al.* 2002).

This very same role is exerted also by another class of bacterial proteins, the members of the Dps family. When discovered in 1992, Dps proteins were found to protect DNA from oxidative damage both *in vitro* and *in vivo* and this protective action was attributed to their interaction with DNA which takes place without apparent sequence specificity. Very recently however Dps proteins were recognized to possess a ferritin-like function that endows them with iron and hydrogen peroxide detoxification properties. This recognition stems to a large extent from the work on *Listeria* ferritin, that was prompted by the observation that no ferritins from Gram-positive bacteria had been characterized and iron availability was known to increase virulence of *L. monocytogenes*, the only pathogenic species for humans. By employing a classical ferritin purification protocol that takes advantage of the very high stability of the apoferritin moiety, quite unexpectedly a 'small' ferritin was isolated, that

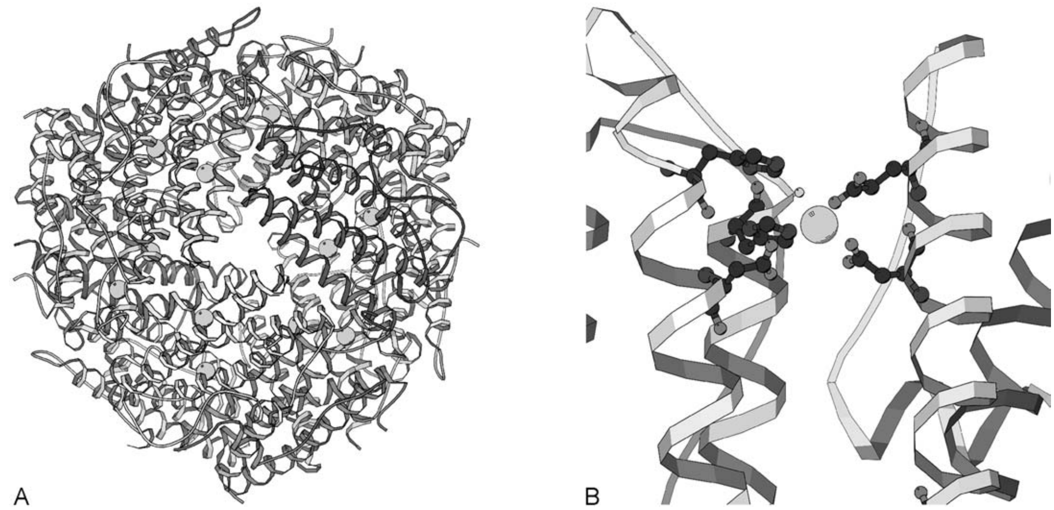

Fig. 1. X-ray crystal structure of *Listeria innocua* ferritin. A: view of the dodecamer along a 3-fold symmetry axis. B: blow up of the intersubunit ferroxidase site showing the iron ligands belonging to two-fold symmetry related subunits.

was assembled from only 12 identical subunits rather than the 'canonical' 24 subunits. Yet this small ferritin was able to oxidize and incorporate iron like a true ferritin. Even more surprisingly, the primary structure did not resemble that of known ferritins, but showed a strong similarity with the Dps proteins (Bozzi *et al.* 1997). The resolution of the X-ray crystal structure (Ilari *et al.* 2000) revealed that this similarity carries over to the quaternary structure, as the protein shell of *Listeria* ferritin displays 32 symmetry (Figure 1A) just like *E. coli* Dps, the family prototype (Grant *et al.* 1998). More importantly, the X-ray structure revealed the presence of a very unusual ferroxidase site that is not located in the four-helix bundle of individual monomers as in all known proteins with ferroxidase activity, but has ligands provided by two symmetry related subunits (Figure 1B). Most of the iron ligands are conserved in the Dps proteins where they are part of the so-called 'DNA-binding signature'. This finding immediately suggested that iron binding could be part of the protective effect exerted by Dps proteins on DNA (Ilari *et al.* 2000). Over the last two years, a number of studies addressed this point and confirmed this contention, namely that the ferritin-like activity is a characteristic of all Dps proteins. Curiously enough, not all the members of the family were found to bind DNA (Papinutto *et al.* 2002, Tonello *et al.* 1999) due to the variability of the N-terminus that in the *E. coli* protein participates in the interaction by means of three lysine residues (Grant *et al.* 1998).

The chemistry of iron oxidation and deposition has been characterized in *E. coli* Dps (Zhao *et al.* 2002). It resembles closely that described for bacterioferritins. Thus, two Fe(II) bind at each of the 12 ferroxidase sites and the pairwise oxidation of Fe(II) is carried out most efficiently by hydrogen peroxide rather than by oxygen, avoiding hydroxyl radical production through Fenton chemistry (reaction 3). Dps acquires a ferric core of about 500 Fe(III)/dodecamer according to the mineralization reaction:

$$2\mathrm{Fe(II)} + \mathrm{H_2O_2} + \mathrm{H_2O} \rightarrow 2\mathrm{Fe(III)OOH_{core}} + 4\mathrm{H}^+$$

again with a 2 Fe(II)/H_2O_2 stoichiometry. In line with the consumption of hydrogen peroxide, spin trapping experiments indicate that Dps greatly attenuates hydroxyl radical production thus avoiding ROS-mediated oxidative damage. The protection of DNA exerted by *E. coli* Dps towards the toxic combination of Fe(II) and hydrogen peroxide can be assessed *in vitro* by means of simple DNA damage assays. Prior to carrying out these assays, the amount of Dps necessary to saturate DNA (400 Dps molecules per plasmid molecule) can be established easily in gel retardation experiments since the *E. coli* Dps-DNA complex does not leave the well (Figure 2). A DNA damage assay is depicted in Figure 3. In the absence of *E. coli* Dps, DNA is fully degraded by the combination of 50 μM Fe(II) plus 10 mM hydrogen peroxide, wheras the presence of saturating amounts of Dps yields essentially complete protection. These results indicate that the protective effect of *E. coli* Dps *in vitro* is exerted by means of a dual action: the physical association

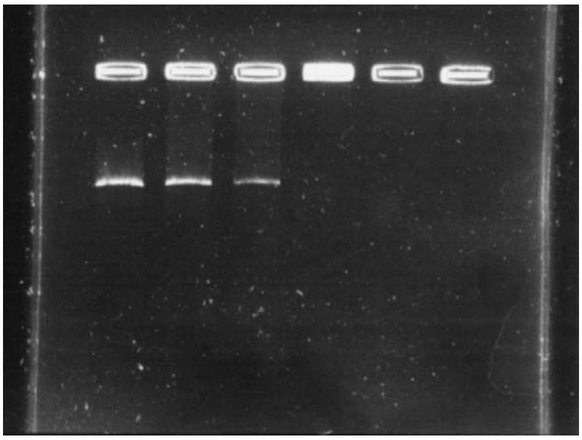

Fig. 2. Binding of *E. coli* Dps to DNA. Linearized pUH21-E DNA (3900 bp, 5 nM) was incubated with *E. coli* Dps in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and was resolved in 1% agarose gel run in Tris-acetic acid-EDTA (TAE) buffer. Lanes (from left to right): 1, pUH21-E DNA (5 nM); 2–4, pUH21-E DNA (5 nM) plus 0.5, 1.0, 2.0 μM Dps, 5–6, empty lanes.

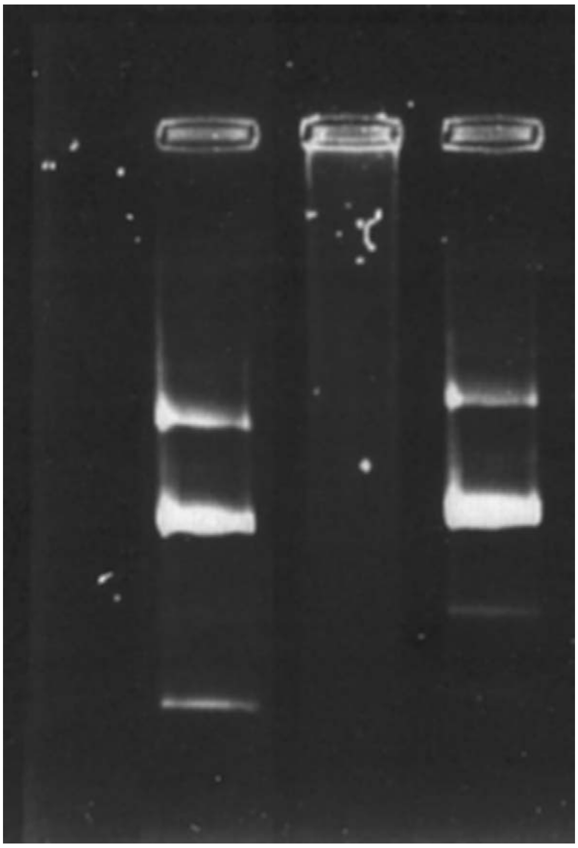

Fig. 3. DNA protection by *E. coli* Dps. Lanes (from left to right): 1, native plasmid pet-11a DNA (5600 bp, 20 nM); 2, plasmid DNA exposed to 50 μM $FeSO_4$ and subsequently to 10 mM H_2O_2; 3, plasmid DNA with 5 μM Dps exposed to 50 μM $FeSO_4$ and subsequently to 10 mM H_2O_2.

with DNA and the ferritin-like capacity to nullify the toxic combination of Fe(II) and hydrogen peroxide (Zhao *et al.* 2002). It is relevant in this connection that in *E. coli* the expression of *dps*, like that of many hydrogen-peroxide inducible activities is regulated by the OxyR transcription factor (Altuvia *et al.* 1994).

Given the fact that not all Dps proteins are able to bind DNA, the natural question is whether Dps proteins confer DNA protection also in the absence of interaction between the two macromolecules. This point was addressed in esperiments on the Dps protein extracted from *Agrobacterium tumefaciens*, a plant pathogen. *A. tumefaciens* Dps does not bind DNA as it does not affect its electrophoretic mobility. Despite the lack of interaction DNA damage assays clearly demonstrates that this Dps protein protects DNA efficiently (Ceci *et al.* 2003). It follows that *in vitro* the iron and hydrogen peroxide detoxification properties of the Dps proteins are the major players in the game.

The *in vivo* relevance of the effects just described is illustrated in very recent studies that bring out also the multiplicity of situations that may be encountered in different bacteria.

The first example concerns the role of Dps in *Campylobacter jejuni*, the microaerophilic Gram-negative bacterium that is a major cause of bacterial diarrhea both in developed and developing countries (Ishikawa *et al.* 2003). In the natural habitat, be it the gastrointestinal tract of the host or the external environment during transmission, *C. jejuni* is likely to encounter iron limitation and oxidative stress, i.e. growth-limiting or potentially lethal conditions. *C. jejuni* contains a 'canonical' ferritin that plays an important role in iron storage under iron-restricted conditions; in addition it possesses a Dps protein that is expressed constitutively. The importance of the iron-detoxifying capacity of this protein in conferring hydrogen peroxide stress resistance is demonstrated by the enhanced sensitivity of a *dps* mutant strain to hydrogen peroxide and by the restoration of resistance achieved by addition of the iron-chelator Desferal in the culture medium. It should be noted that *C. jejuni* Dps does not bind DNA.

An interesting case is provided by *Streptococcus mutans* (Yamamoto *et al.* 2002). Like all lactic acid bacteria, *S. mutans* can not synthesize heme and therefore lacks cytochrome oxidases required for energy-linked oxygen metabolism as well as catalase and heme-peroxidase required for oxygen tolerance. However, *S. mutans* like many lactic acid bacteria can grow in the presence of oxygen and is even able to consume oxygen due to the action of flavoenzymes. *S. mutans* does not contain 'canonical' ferritins nor bacterioferritins, but expresses a Dps protein named Dpr, for **D**ps-like **p**eroxide-**r**esistance, since it protects cells from peroxides and hence has an indispensable role in conferring oxygen tolerance. This is demonstrated by the fact that *dpr* disruption mutants in the presence of air show a decrease of about 10^4-fold in the number of colonies formed compared to the wild-type strain.

The Gram-negative obligate anaerobe *Porphyromonas gingivalis*, which is strongly associated with chronic periodontitis, likewise provides an interesting case (Ueshima *et al.* 2003). This bacterium expresses numerous potential virulence factors (e.g. fimbriae, hemagglutinins, lipopolysaccharides) and various proteases that are able to hydrolyze a number of host proteins. By definition *P. gingivalis* can not grow in aerobic conditions, but exhibits a high degree of aerotolerance. It lacks catalase, but possesses a 'canonical' ferritin (Ftn) and a Dps protein whose expression is significantly increased in an OxyR-dependent manner upon exposure of the bacterium to air. Interestingly, *dps, ftn* and *dps ftn*-deficient mutants are as tolerant to atmospheric oxygen as the wild type strain. However, the Dps protein confers hydrogen peroxide resistance as apparent from the increased sensitivity of the *dps* and *dps ftn*-deficient mutants to hydrogen peroxide relative wild type and the *ftn* deficient strain in the presence of air. Dps also plays a role in the survival of *P. gingivalis* in infected cells as shown by the decrease of viability in human umbelical vein endothelial cells of the *dps* deficient mutant as compared to wild type, although there was no significant difference in invasion between these strains.

In conclusion, whereas in the extracellular medium the relationship between iron, microorganisms and the host is governed essentially by a 'battle of chelating agents' i.e. by the competition between lactoferrin and bacteria for iron, in the bacterial cell the situation is quite diversified. Not only the number and type of iron-transport and -uptake systems may differ, but also the number and type of iron storage and detoxification proteins. *E. coli* is prototypical of bacteria which acquire iron by means of a multiplicity of iron transport systems and possess a number of different proteins for iron storage and detoxification, namely two 'canonical' ferritins, a bacterioferritin and a Dps protein, whereas *L. innocua* or *S. mutans* exemplify bacteria that have relatively few iron acquisition systems and possess only one iron storage/detoxification protein, namely Dps. This is suggestive of a parallelism between the multiplicity of iron storage/detoxification systems and the multiplicity of iron acquisition systems that leads to a much more complex interplay between iron, microorganisms and the host than hitherto believed.

Acknowledgements

This paper is dedicated to the memory of our unforgettable mentor Professor Eraldo Antonini, deceased prematurely on March 19, 1983, who introduced us to the study of iron transport and storage proteins.

This work was supported in part by grants from the Istituto Pasteur-Fondazione Cenci Bolognetti (to E. C.) and from the Ministero Istruzione Universitá e Ricerca, Project 'Stress ossidativo e metalloproteine in infezioni da batteri intracellulari facoltativi' (to S. S.) and local funds (to E. C.).

References

Almiron M, Link AJ, Furlong D, Kolter R. 1992 A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli. Genes Dev* **6**, 2646–2654.

Altuvia S, Almiron M, Huisman G, Kolter R, Storz G. 1994 The dps promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. *Mol Microbiol* **13**, 265–272.

Andrews SC. 1998 Iron storage in bacteria. *Adv Microb Physiol* **40**, 281–351.

Bou-Abdallah F, Lewin AC, Le Brun NE, Moore GR, Chasteen ND. 2002 Iron detoxification properties of *Escherichia* coli bacterioferritin. Attenuation of oxyradical chemistry. *J Biol Chem* **277**, 37064–37069.

Bozzi M, Mignogna G, Stefanini S, Barra D, Longhi C, Valenti P, Chiancone E. 1997 A novel non-heme iron-binding ferritin related to the DNA-binding proteins of the Dps family in *Listeria innocua J Biol Chem* **272**, 3259–3265.

Ceci P, Ilari A, Falvo E, Chiancone E. 2003 The Dps protein of *Agrobacterium tumefaciens* does not bind to DNA, but protects it towards oxidative cleavage. X-ray crystal structure, iron binding and hydroxyl-radical scavenging properties. *J Biol Chem* **278**, 20319–20326.

Cheesman M *et al.*1993 Haem and non-haem iron sites in *Escherichia coli* bacterioferritin: spectroscopic and model building studies. *Biochem J* **292**, 47–56.

Cornelissen CN, Sparling PF 1994 Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. *Mol Microbiol* **14**, 843–50.

Deneer HG, Healey V, Boycluk I. 1995 Reduction of exogenous ferric iron by a surface-associated ferric reductase of *Listeria* spp. *Microbiology* **141**, 1985–1992.

Douglas T, Ripoli DR 1998 Calculated electrostatic gradients in recombinant human H-chain ferritin. *Protein Sci* **7**, 1083–91.

Frolow F, Kalb AJ (Gilboa), Yariv J. 1994 Structure of a unique twofold symmetric haem-binding site. *Nat Struct Biol* **1**, 453–460.

Grant RA, Filman DJ, Finkel SE, Kolter R, Hogle JM 1998 The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat Struct Biol* **5**, 294–302.

Harrison PM, Arosio P. 1996 The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* **1275**, 161–203.

Ilari A, Stefanini S, Chiancone E, Tsernoglou D 2000 The dodecameric ferritin from *Listeria innocua* contains a novel intersubunit iron-binding site. *Nat Struct Biol* **7**, 38–43.

Ishikawa T, Mizunoe Y, Kawabata S, Takade A, Harada M, Wai SN, Yoshida S. 2003 The iron-binding protein Dps confers hydrogen peroxide stress resistance to *Campylobacter jejuni. J Bacteriol* **185**, 1010–1017.

Johnson WL, Varner L, Poch M. 1991 Acquisition of iron by *Legionella pneumophila*: role of iron reductase. *Infect Immun* **59**, 2376–2381.

Jones T, Spencer R, Walsh C. 1978 Mechanism and kinetics of iron release from ferritin by dihydroflavins and dihydroflavin analogues. *Biochemistry* **19**, 4011–4017.

Papinutto E, Dundon WG, Pitulis N, Battistutta R, Montecucco C, Zanotti G. 2002 Structure of two iron-binding proteins from *Bacillus anthracis. J Biol Chem* **277**, 15093–15098.

Schryvers AB, Stojilijkovic I. 1999 Iron acquisition systems in the pathogenic *Neisseria. Mol Microbiol* **32**, 1117–1123.

Takagi H, Shi D, Ha Y, Allewell NM, Theil EC. 1998 Localized unfolding at the junction of three ferritin subunits. A mechanism for iron release? *J Biol Chem* **273**, 18685–18688.

Tonello F, Dundon WG, Satin B, Molinari M, Tognon G, Grandi G, Del Giudice G, Rappuoli R, Montecucco C. 1999 The Helicobacter pylori neutrophil-activating protein is an iron-binding protein with dodecameric structure. *Mol Microbiol* **34**, 238–246.

Ueshima J, Shoji M, Ratnayake DB, Abe K, Yoshida S, Yamamoto K, Nakayama K. 2003 Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis. Infect Immun* **71**, 1170–1178.

Wandersman C, Stojiljkovic I. 2000 Bacterial heme sources: the role of heme, hemporotein receptors and hemophores *Curr Opin Microbiol* **3**, 215–220.

Yamamoto Y, Poole LB, Hantgan RR, Kamio Y. 2002 An iron-binding protein, Dpr, from Streptococcus mutans prevents iron-dependent hydroxyl radical formation *in vitro. J Bacteriol* **184**, 2931–2939.

Yang X, Chen-Barret Y, Arosio P, Chasteen ND. 1998 Reaction paths of iron oxidation and hydrolysis in horse spleen and recombinant human ferritins. *Biochemistry* **37**, 9743–9750.

Zhao G, Ceci P, Ilari A, Giangiacomo L, Laue TM, Chiancone E, Chasteen ND. 2002 Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli. J Biol Chem* **277**, 27689–27696.

Zhao G, Bou-Abdallah F, Arosio P, Levi S, Janus-Chandler C, Chasteen ND. 2003 Multiple pathways for mineral core formation in mammalian apoferritin. The role of hydrogen peroxide. *Biochemistry* **18**, 3142–3150.