

Expression and regulation pattern of ferritin-like DpsA in the archaeon *Halobacterium salinarum*

Sabine Reindel¹, Christian L. Schmidt², Stefan Anemüller² & Berthold F. Matzanke^{1,*}

¹Isotopenlabor TNF, Universität zu Luebeck, Ratzeburger Allee 160, D-23538, Luebeck, Germany; ²Institute of Biochemistry, University of Luebeck, 23 538, Luebeck, Germany; * Author for correspondence (Tel.: +49-451-5004140; Fax: +49-451-5004139; E-mail: matzanke@physik.uni-luebeck.de)

Key words: Dps, ferritin, iron metabolism, transcription, transcription binding factor

Abstract

Very recently, an iron-rich protein, DpsA, was isolated from the extreme halophilic euryarchaeon *Halobacterium salinarum* JW5 and characterized. The amino acid sequence of DpsA is related to Dps proteins which belong structurally to the ferritin superfamily but differ from ferritins in their function and regulation. Employing Northern and Western blot analysis, the expression of DpsA in *H. salinarum* was examined throughout all growth phases and under a variety of growth conditions (iron deficiency, iron supplied growth, oxidative stress). DpsA shows increasing expression of *dpsA* mRNA in iron rich media and under conditions of oxidative stress (H₂O₂), whereas under iron deficient conditions mRNA-levels decrease. This is in contrast to Dps-type proteins the transcription of which is induced under conditions of iron starvation. Northern blot experiments show that the expression pattern of halobacterial DpsA is the same as that found in the few bacterial non-heme ferritin the expression pattern of which has been analyzed so far. Based on Western-blot analysis post-transcriptional regulation, typical of mammalian ferritins, can be excluded. This protein exhibits features of a non-heme type bacterial ferritin although it shares only little sequence similarity with Ftn from *E. coli*.

Abbreviations: Dps – DNA binding protein from starved cells; Bfr – heme containing bacterial ferritin; Ftn – non-heme bacterial ferritin

Introduction

Within a major project analyzing archaeobacterial iron transport and iron metabolism we have focussed our interest on possible iron storage forms in the euryarchaeon *Halobacterium salinarum*. *H. salinarum* is extremely halophilic requiring 4.2 M NaCl for optimal growth. To cope with osmotic stress *H. salinarum* accumulates up to 5 M K⁺-Ions in its cytosol. This chemoorganotrophic, obligate aerobic organism (Tindall 1992) exhibits highly acidic cytoplasmic proteins and is, therefore, excellently adapted to live in an environment of high ionic strength (Lanyi 1974).

Recently, we characterized a novel iron-containing protein from the *H. salinarum* (Reindel *et al.* 2002). This protein exhibits typical features of a ferritin: it is heat stable, the molecular mass of each subunit is 20 kDa, the subunit shows a highly α -helical secondary structure and subunits assemble to a hollow spherical shell where the iron atoms can be deposited. Heme groups as observed in Bfr-type bacterioferritins are not present. In general, the protein shell of a ferritin is built up by 24 subunits. For our isolated protein, however, a dodecameric assembly with a single subunit-type could be derived. The peptide sequence of fragments of that protein were compared with a

genome derived protein library of *H. salinarum* sp. NRC-1 disclosing an identity of 100% with DpsA (DNA binding protein from starved cells). Further sequence alignments uncovered partial identities with the DpsA protein from *Synechococcus* sp. and with the non-heme ferritins (Ftn) from *Listeria innocua* and from *Helicobacter pylori* (named NapA). Both non-heme ferritins exhibit dodecameric structures, too. Employing molecular modelling of the halobacterial DpsA protein a four-helix-bundle structure was obtained with one additional smaller α -helix connecting helix B with helix C. In typical subunits of 24-meric ferritins, however, the small α -helix is located at the C-terminal end. In addition, halobacterial DpsA is lacking the typical intra-subunit-ferroxidase site which catalyzes the oxidation of Fe^{2+} to Fe^{3+} in eukaryotic H-chain and in tetraicosameric bacterial ferritins. This type of ferroxidase site is also missing in other dodecameric ferritins, for example in the non-heme ferritin of *H. pylori* (Tonello *et al.* 1999; Zanotti *et al.* 2002). In NapA from *H. pylori* the ferroxidase site is located at the interface of two subunits (Evans Jr. *et al.* 1995; Tonello *et al.* 1999).

Surprisingly, all Dps proteins characterized so far (Grant *et al.* 1998; Ilari *et al.* 2000; Yamamoto *et al.* 2000; Gupta *et al.* 2002; Papinutto *et al.* 2002; Ceci *et al.* 2003; Ishikawa *et al.* 2003; Pulliainen *et al.* 2003; Ren *et al.* 2003) show the same properties as the new class of dodecameric ferritins: (i) subunits exhibit a molecular mass of approximately 20 kDa, (ii) the subunit tertiary structures consist of a four α -helix bundle with one additional smaller α -helix connecting helix B with helix C, (iii) six or 12 subunits assemble to a hollow spherical shell, (iv) a ferroxidase site is present located at the interface of two subunits and (v) with the exception of DpsA from *Synechococcus* (Pena & Bullerjahn 1995a; Pena *et al.* 1995b) – no heme groups are detectable.

On the other hand, differences exist on a regulatory and on a functional level. Some Dps proteins show non-specific DNA binding (Azam *et al.* 2000; Chen & Helmann 1995). The abundance of the mRNA of *dpsA*_{*Synechococcus*}/*mrgA*_{*B. subtilis*}/*non-heme ferritin*_{*L. innocua*} is low under conditions of iron-rich growth whereas an induction is observed under conditions of iron starvation (Chen *et al.* 1993; Altuvia *et al.* 1994; Polidoro *et al.* 2002). The expression is also induced in response

to oxidative stress (Chen & Helmann 1995; Sen *et al.* 2000). Although the structures and catalytic functions of bacterial ferritins are well known, merely limited data on their regulation in bacteria are available. For Bfr (bacterioferritin) and FtnA from *E. coli* and for Pfr of *H. pylori* an accumulation is observed under iron-rich growth conditions in the stationary phase. In contrast to *dps* regulation the expression of bacterial ferritins is low under conditions iron starvation (Abdul-Tehrani *et al.* 1999; Bereswill *et al.* 2000).

The ability of Dps proteins to bind DNA non-specifically is not proven for all members of this class of proteins and it is very difficult to examine protein–DNA interactions at the existing high salt concentration in the cytosol of *H. salinarum*. In order to decide whether halobacterial DpsA is functionally a Dps or a bacterial ferritin we examined the expression of the protein under different growth conditions by Northern and Western blot analysis throughout all growth phases. Based on our observations, we conclude that the regulation of halobacterial DpsA differs from that of other Dps proteins. Rather, regulation seems to be similar to what is known about bacterial ferritin regulation and the general archaeal transcription initiation.

Materials and methods

Organism and growth conditions

H. salinarum (DSM 3754), strain JW 5, was a kind gift of Dr. Peter Palm (MPI für Biochemie, Martinsried Germany). This strain is defective in carotenoid-biosynthesis and consequently can not grow by converting light into energy. Therefore, aerobic growth is dependent on a functional respiratory chain. The iron rich medium contained: 4.2 M NaCl, 81 mM MgSO_4 , 10 mM Na-citrate, 26.8 mM KCl, 200 μM FeCl_3 , 10 g neutralized bacteriological peptone L34 (Oxoid), pH 7.2. Iron limited medium contained the same concentrations of NaCl, Na-citrate, KCl and peptone. This solution was incubated with Chelex® 100 resin (BioRad, 5 g/100 ml) over night to remove traces of iron. After decanting this solution 81 mM MgSO_4 and an appropriate volume of a 1000 fold concentrated trace elements solution (containing per liter: 9 mM MnCl_2 ,

11.8 mM $\text{Na}_2\text{B}_4\text{O}_7$, 1.6 mM ZnSO_4 , 0.29 mM CuCl_2 , 0.12 mM Na_2MoO_4 , 0.12 mM VOSO_4 , 0.036 mM CoSO_4) were added to the supernatant. To eliminate iron contaminations glass ware was treated in succession with alkaline methanol (1 N KOH, 1:1 methanol/ $\text{H}_2\text{O}_{\text{bidest}}$), 2 N HCl and 50 mM EDTA. In order to produce oxidative stress 1 mM H_2O_2 (final concentration) was added after 24 h of growth to the iron rich and iron limited medium. All growth media were inoculated per 100 ml with 1 ml of an iron depleted cell suspension ($\text{OD}_{500} = 1.17$). The cells were grown aerobically in a gyratory shaker at 100 rpm and 37 °C. Aliquots for total RNA- and cytosol-preparations were taken at 16, 24, 36, 48, 60, 84 h of growth.

Total RNA isolation and Northern blot analysis

Total RNA isolation was performed as described (Chomczynski & Sacchi 1987). Total RNA (5 μg) was electrophoresed in a 1.2% formaldehyde/agarose gel and transferred on a nylon membrane (HybondTM-N, Amersham) as described in (Chomczynski 1992). In order to normalize the *dpsA* signal intensity with reference to the general transcription rate of the cell we chose the 16S rRNA signal intensity as reference. Therefore, probes for the *dpsA* mRNA and the 16S rRNA were generated with the nested PCR method (Poster-Jordan *et al.* 1990) using the primers listed in Table 1. After prehybridization and random primed labelling reaction the *dpsA* probe was added to the blot over night. The blot was exposed to a phosphorus-

imaging screen and after a second prehybridization the 16S rRNA probe was added to the same blot. By variation of the exposure times it was possible to prevent an intensity-increase of the Northern blots beyond the linear detection-range of the screens. Intensity-determinations of single bands were achieved using a phosphoimager (BAS1000, raytest)-and the AIDA program (Version 2.11, copyright by raytest). The intensity data of *dpsA* were normalized taking into account exposure time, the degree of labelling of the probe and the integral density of the 16S rRNA signal. To check the *dpsA* mRNA and the 16S rRNA size, the distances of the RNA ladder of the formaldehyde/agarose gel was transcribed to the Northern blot.

Polyclonal antibodies

Polyclonal antibodies against the native and denaturated *H. salinarum* DpsA were generated in a rabbit. The protein was purified as described recently (Reindel *et al.* 2002). In bimonthly intervals, a Chinchilla bastard rabbit was immunized subcutaneously with a solution containing 500 μg heat denaturated protein (10 min, 100 °C), 500 μg native protein, and an equal volume of incomplete Freund's adjuvant (Sigma). Peripheral blood was collected and stored for use as polyclonal antiserum.

Western blot analysis

Cells were grown aerobically in a shaker at 100 rpm and 37 °C for 16, 24, 36, 48, 60, 84 h, respectively (conditions as described above). Cytosol of each sample was prepared as described in (Sreeramulu *et al.* 1998). Protein concentrations were determined based on the Lowry method employing the Bio-Rad D_c protein assay. Amounts of 5 μg total protein were electrophoresed in a 4–12% SDS-PAGE using the ready-to-use NuPAGE[®] electrophoresis system (Invitrogen) and transferred on a nitrocellulose filter (45 μm , Schleicher & Schüll) using the NuPAGE[®] Western transfer apparatus (Invitrogen). DpsA was detected by using a 1:200 dilution of the rabbit serum which contained polyclonal antibodies against native and denaturated DpsA. The immune reaction was visualized with the Novex chromogenic Western blot immunodetection kit WesternBreeze[®] (Invitrogen).

Table 1. Primers. Primers for the preparation of *dpsA* and 16S rRNA probes.

Name	Sequence
dpsA sen1	CGT TAT GAG CAG GCA AAA GA
dpsA anti1	AAC GCT AGT CGC GAA AGA A
dpsA sen2	CGC CGA ACA GTG TGT AGA
dpsA anti2	TCG TCT TCG AGC TCG ATG
16S rRNA sen1	ATT GCT ATC GGA GTC CGA TTT A
16S rRNA anti1	GGC TAC CTT GTT ACG ACT TAA T
16S rRNA sen2	GGC ATA GAG CCT TCA CTT TT
16S rRNA anti2	GTC AGC TGA ACA GTT T

In silico analysis

In order to investigate possible promoter binding sites nucleotide sequence alignments were generated with the FASTA program (<http://pir.georgetown.edu/>).

Results

Growth conditions of H. salinarum

Suspensions of *Halobacterium salinarum* were cultured under four distinct conditions: iron-rich growth ([Fe] approx. 200 μ M), iron-rich growth under oxidative stress, iron limited growth ([Fe] ≤ 0.5 μ M in the medium) and iron-limited growth under oxidative stress. Oxidative stress was induced by adding H₂O₂ (1 mM final concentration) after 24 h of growth cell suspensions. The applied amount of H₂O₂ did not show any effect on halobacterial growth rates (Figure 1). Typically, growth rates of iron limited cultures were half of the growth rates of iron-rich cultures. However, it was not possible to starve out this organism by serial subcultures grown under low-iron conditions.

Northern blot analysis

Northern blot analyses of the four different cultures described above performed with total RNA iso-

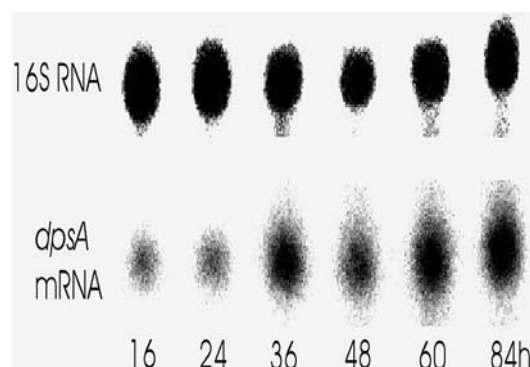


Figure 2. Example of a Northern blot performed with total RNA isolated from iron-rich cultures taken at various times of growth. Row one shows the transcription of the 16S rRNA, row two displays the transcription of halobacterial *dpsA*. There were no cross-hybridizations of the *dpsA* and 16S rRNA probe (data not shown).

lated from six aliquots taken at various times during incubation. Figure 2 shows a typical Northern blot for iron rich growth conditions. Each *dpsA* mRNA signal was normalized to the signal of the 16S rRNA. Figure 3 displays the evaluation of the different Northern blots. Each experiment was performed three times with independent cultures. The analysis reveals a decrease of the abundance of the *dpsA* mRNA in the late log phase for iron limited growth. This decrease starts after 36 h of growth at 0.4% integral density and reaches a level of 0.16% \pm 0.03% integral density at 84 h of growth.

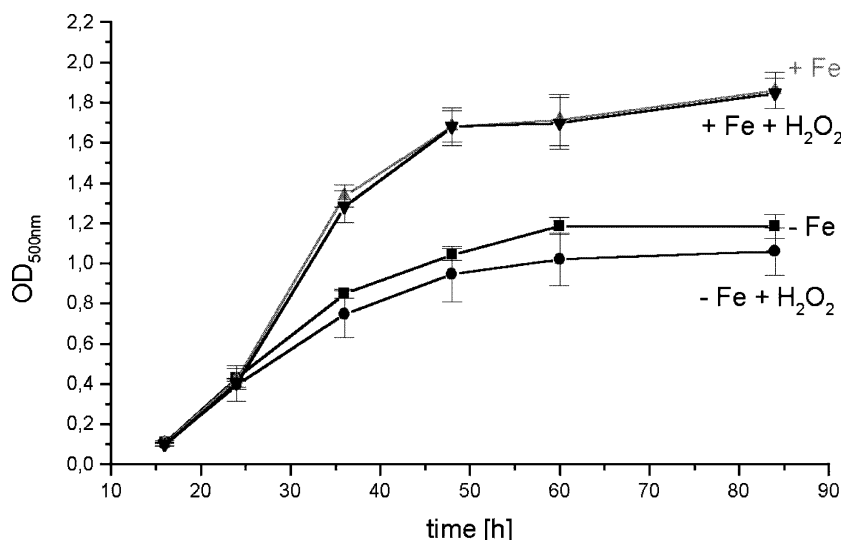


Figure 1. Growth curves of four independent cultures of the different growth conditions. Grey triangles (\blacktriangle) correspond to iron-rich growth conditions ([Fe] in the medium: 200 μ M); black (\blacktriangledown) iron-rich growth under conditions of oxidative stress (1 mM H₂O₂); black squares (\blacksquare): iron deficient medium ([Fe] < 0.5 μ M); dark grey circles (\bullet): iron deficient medium and oxidative stress (1 mM H₂O₂). Composition of the media is described in Materials and methods.

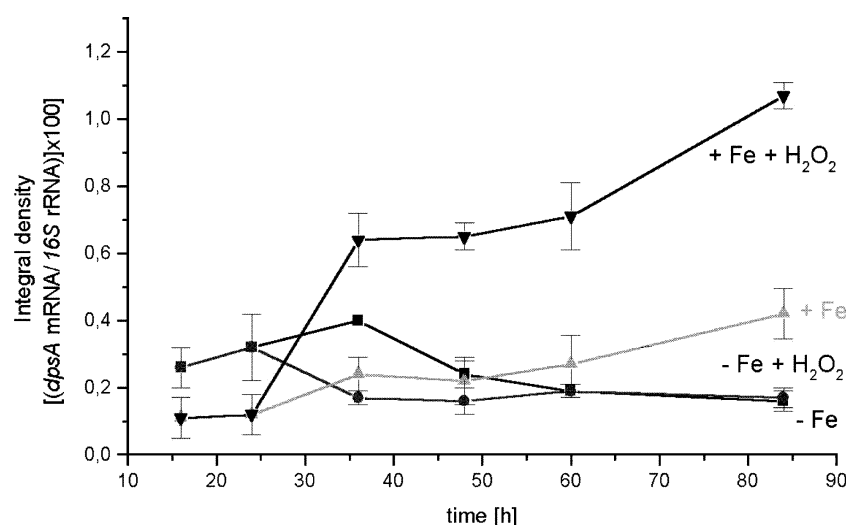


Figure 3. Graph of Northern blot analysis at four different growth conditions. *H. salinarum* – RNA was prepared at various points of time during growth, subjected to Northern blotting, and hybridized with the *dpsA* and *16S rRNA* probe, respectively. Transcription of the *16S rRNA* was used as an internal control to correct RNA loading and to calculate the relative transcription level of the *dpsA* gene for each condition and time. Each experiment was performed three times with independent cultures. Variations are indicated by error bars. Black triangles (▼) correspond to probes isolated at the corresponding time grown under iron-rich conditions and oxidative stress (1 mM H₂O₂). Grey triangles (▲) correspond to iron-rich growth conditions; black squares (■): iron deficient medium ([Fe] < 0.5 μM); dark grey circles (●): iron deficient medium and oxidative stress (1 mM H₂O₂).

A decrease is also observed for iron limited growth under oxidative stress. This decrease starts immediately after adding H₂O₂. At 24 h of growth the percentage of the relative integral density amounts to $0.32 \pm 0.1\%$, whereas after 36 h of growth this value decreases to $0.17\% \pm 0.02\%$. Iron limitation under oxidative stress stops enhanced expression of the halobacterial *dpsA* already in the log phase. Under condition of iron-rich growth the expression increases continuously from $0.11 \pm 0.06\%$ at 16 h to $0.42 \pm 0.075\%$ integral density at 84 h. Under conditions of iron-rich growth in combination with oxidative stress the expression increases within 12 h after addition of H₂O₂ from $0.11 \pm 0.06\%$ up to $1.07 \pm 0.04\%$ integral density. The overall pattern of the expression displays an upregulation of the halobacterial *dpsA* mRNA under iron-rich growth conditions and a downregulation under condition of iron starvation. Oxidative stress enhances this effect especially under iron-rich growth conditions. This regulation behaviour is in contrast to what is known for *dps* regulation but is in line with bacterial *ferritin* regulation.

Immunoblots

Western blot analyses (Figure 4) were performed with cytosol isolated from six aliquots taken at

various times during growth of any of the four different cultures described above. Blots reveal an increasing signal with time under all growth conditions tested. Pre-immuneserum (zero-blood) was used as a control. As expected, no reaction was observed (data not shown). In the Western blots we observed a stepwise denaturation of DpsA resulting in multiple bands per lane. Interestingly, the most intense band on the cytosol immunoblots is found at 40 kDa, corresponding to the dimeric form of DpsA. The second most intense signal occurs at 20 kDa corresponding to the monomer. Weaker signals in 20 kDa steps are also visible. This is a result of a stepwise denaturation of the protein forming stable dimeric, trimeric and higher oligomeric aggregates of DpsA, with the dimeric form as the main denaturation product under the conditions of high salinity of the cytosol. This observation is in line with the denaturation behavior of 24-meric ferritins (Listowsky *et al.* 1972). In addition, there was a discrepancy in the apparent molecular masses of the monomer in the Western blots (21 kDa) versus SDS-PAGE (25 kDa) (Reindel *et al.* 2002). Furthermore, a Western blot performed with purified DpsA showed a single band with an apparent molecular mass of 25 kDa. This is caused by the high salt concentration of the halobacterial cytosol taken for the Western blots

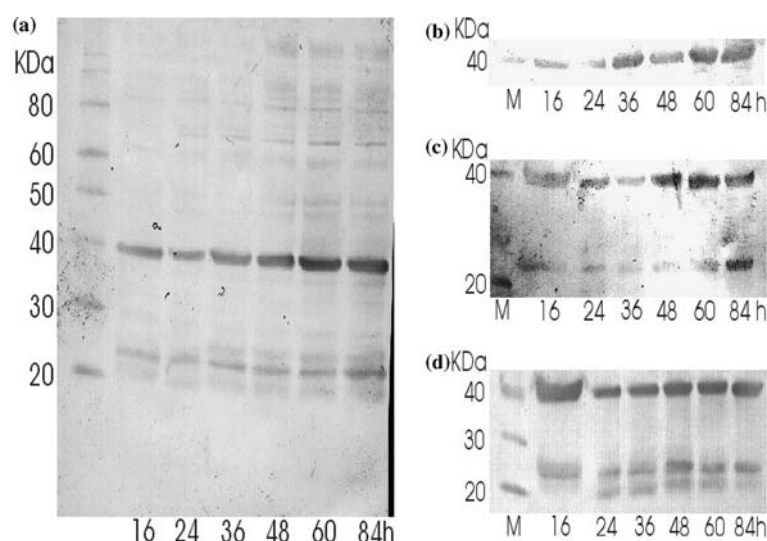


Figure 4. Western blots at four different growth conditions. Growth conditions are the same as described in Figure 3. Cytosol of *H. salinarum* was prepared at various points of time during growth, subjected to Western blotting, and detected by using a 1:200 dilution of polyclonal antibodies against ferritin. Only blot (a) is shown in full size, because the overall pattern of the blots is nearly identical. A size marker (MagicMark, Invitrogen) is shown to the left. (a) Iron-deficient growth (b) iron-deficient growth under oxidative stress (c) Iron-rich growth (d) Iron-rich growth under oxidative stress.

whereas on SDS PAGE salt-free DpsA was analyzed. The high salt concentration seems to stabilize the oligomeric structure of DpsA.

In silico analysis

Since the complete genome sequence from *H. salinarum* sp. NRC-1 is known (Ng *et al.* 2000) and available at NCBI (Acc. No.: NP_281044), we perform a sequence alignment of the region 210 bp upstream of the *dpsA* gene with sequences of known transcription factor binding motives. Five different promoter are known in promoter regions of genes involved in the regulation of iron homeostasis and of *dps* (Figure 5): (i) Fur box (Ferric-uptake regulator; consensus sequence from *E. coli*), (ii) Fur box (Ferric-uptake regulator; consensus sequence from *H. pylori*), (iii) DtxR (diphtheria-toxin regulator, consensus sequence from *C. diphtheriae*), (iv) PerR (consensus sequence from *B. subtilis* and *S. aureus*), and (v) the OxyR binding site from *E. coli*.

In bacteria two iron regulatory repressors are known: Fur and DtxR. The physiological role of Fur and DtxR is similar. They are metal-activated transcriptional repressors found in gram-negative and gram-positive bacteria, respectively, participating in the control of iron homeostasis. Pfr, the

24-meric ferritin from *H. pylori*, is the sole representative of its class known to be directly controlled by Fur (Waidner *et al.* 2002). The Dps protein from *B. subtilis*, MrgA, is under control of PerR, a peroxide inducible, iron dependent repressor (Fuangthong *et al.* 2002). Dps from *E. coli* is under control of OxyR, a hydrogen peroxide response regulator (Altuvia *et al.* 1994).

The analysis of the upstream DNA sequence of the *dpsA* gene discloses a true promoter region halobacterial TATA box: NTTTTWWN (W = A or T; N = A, T, G or C) (Soppa 2001). Moreover, the transcription factor element B (BRE) upstream to the TATA box could be identified (Soppa 2001) (Figure 5), upstream sequence alignments revealed the highest identity with the DtxR consensus sequence from *C. diphtheriae* (68.4% identity in a 19 nt overlap). For the PerR consensus sequence from *B. subtilis* and *S. aureus* 66.7% identity in a 15 nt overlap was found, 63.2% identity in a 19 nt overlap with the Fur box consensus sequence from *E. coli*, 58.6% identity in a 29 nt overlap with the OxyR binding sequence from *E. coli* and 45% identity in a 20 nt overlap with the Fur box from *H. pylori*.

These sequence alignments reveal possible transcription factor binding sites indicating that the halobacterial *dpsA* very likely is under the

```

-210 CGATCATCGG TGTGGCGTAG TGTGTGGGGG CGTGTGAGCG TTGCGGCCGG TGGTGAGGCC CACTCGGCTG TGCCTGGTGT
1) ACGCTTGTTA CCACTATTAG TGTGATAGGA ACAGCCAGAA TAGCGGAACA

-130 TTGGGTGGCC CCGAGTACGC CATCACAGAG AATAACAACG GGTATCGAAG AAATTGTGTT TTGGTACGCC AAACAAAATT
2) GAT AATGATAATC ATTATC 3) TATN ANNNNNTTTT AATNAAAATN
4) T TAGGTTAGCC TAACCTAA

-6 -3 M S
- 50 TGGATGCAGC GGAAAGCTTT AAGTGGCAGG CCGTCCAACG TGTAATCGTT ATG AGC
3) ATAAAAANATT 5) T TATAATNATT ATA

```

Figure 5. *In silico* analysis with possible promoter binding sites of different promoters. (1) *E. coli* OxyR binding box (58.62% identity in 29 nt overlap) (Altuvia *et al.* 1994). (2) *E. coli* Fur box consensus sequence (Delany *et al.* 2001) (63.16% identity in 19 nt overlap). (3) *H. pylori* Fur box consensus sequence (45% identity in 20 nt overlap) (Delany *et al.* 2001). (4) *C. diphtheriae* DtxR consensus sequence (68.42% identity in 19 nt overlap) (Quian *et al.* 2002). (5) *B. subtilis* and *S. aureus* PerR consensus sequence (66.67% identity in 15 nt overlap) (Mongkolsuk & Helmann 2002). Boxed nucleotides indicate the TATA-box which is required for transcription of all types of genes. Numbers above nucleotides indicate the position of the Transcription factor B element upstream of the TATA-box. The underlined triplet indicates the transcription start and the capitals above the triplets indicate the beginning of the *ferritin* gene (Soppa 2001).

control of iron regulatory repressors and/or under the control of oxidative stress repressors.

Discussion

From the growth experiments under conditions of iron-deficiency carried out in this study it can be derived that it is not possible to starve out *H. salinarum* by serial low-iron subcultures. We assume that – like in *E. coli* (McHugh *et al.* 2003) – *H. salinarum* is able to switch gene expression to alternative systems when iron starvation starts. For example, the respiratory chain is expressed under conditions of iron starvation in a way requiring only very small amounts of iron (Hubmacher *et al.* 2003). Another strategy might be a change in the biosynthesis of ribonucleotide reductase going from the synthesis of a class-I to a class-II enzyme. Class-I ribonucleotide reductases possess iron, whereas class II reductases employ vitamin B₁₂ as a cofactor. Homologies to both classes are found in the halobacterial genome (Sjöberg, personal communication.)

Almost all organisms require iron to sustain a wide variety of biological reactions but on the other hand iron is also a potentially toxic element. As a consequence organisms have developed a very balanced and complex regulatory system for iron metabolism and, in particular, for iron homeostasis. The objective of regulation is to minimize the internal “free” iron content in the cell. This is achieved mainly on two levels: (i) by

up- or down-regulation of iron-uptake systems depending on the iron deprivation state of the cell (transferrin, siderophores, membrane-transport proteins) and (ii) by up - or down-regulation of the iron storage protein ferritin, depending on the intracellular availability of iron. The latter process is very well characterized in eukaryotic systems by the IRE/IRP post-transcriptional regulatory system (Torti *et al.* 2002). In prokaryotes, however, much less is known about regulation of bacterial ferritins, whereas the regulation of iron transport into the cell by the Fur- (Gram-negative bacteria) and DtxR-regulator (Gram-positive bacteria) (Escobar *et al.* 1999; Hantke 2001) is well characterized. To our knowledge there exists merely one example of a promoter regulated ferritin gene, the Pfr from *H. pylori* (Bereswill *et al.* 2000). Transcription of *pfr* is iron-induced and Fur-dependent (Van Vliet *et al.* 2002) but synthesis of *pfr* mRNA is completely repressed under conditions of iron starvation (Bereswill *et al.* 2000). Transcription of *napA* (neutrophil activating protein), the 12-meric non-heme ferritin from *H. pylori*, showing significant sequence and structural homologies to our protein, is neither affected by iron nor by Fur (Van Vliet *et al.* 2002). For *E. coli*, it was shown that Ftn and Bfr expression is increasing concomitant to an increasing iron content of the cell (Abdul-Tehrani *et al.* 1999). Recent work revealed that the expression of Ftn is lacking in a *fur* negative mutant (McHugh *et al.* 2003) – although a Fur binding site could not be identified. For *E. coli*, it is assumed that the *fur* dependent regulation of

Ftn in is achieved via a small non-coding RNA, named RhyB (Massé *et al.* 2002). The Northern blot analysis of the halobacterial *dpsA* mRNA in the present study shows an increase of transcription up to 0.42% integral density in iron-rich media, whereas in iron limited media the transcription decreases leading to 0.16% integral density in the stationary growth phase. This is in line with the regulation of bacterial ferritin as far as it is known today.

Iron homeostasis is linked to oxidative stress via damage of iron-sulfur clusters (Imlay 2002). As a result an increased level of “free” iron occurs in the cell generating increased levels of hydroxyl radicals by Fenton chemistry, which in turn intensify oxidative stress. Therefore, one should expect, that the regulation of ferritins is affected by oxidative stress. However, a direct regulatory link has been observed so far only in eukaryotes. There, it was shown that cells overexpressing ferritin are more resistant to oxidative stress and that under stress conditions the ferritin expression is stimulated in a complex way by cytokines (Testa 2002). In *E. coli* and *Campylobacter jejuni* absence of ferritin increases the sensitivity to oxidative stress. For a ferritin-deficient mutant of *C. jejuni*, was complemented with a plasmid encoding bacterial ferritin, it is assumed, that the observed resistance against oxidative stress was achieved by sequestering iron via ferritin (Andrews 1998).

A decade ago a new class of proteins, named Dps, was discovered. The first representative of this class was isolated from *E. coli* (Almirón *et al.* 1992). *Dps* transcription is induced during exponential growth by OxyR (Altuvia *et al.* 1994). OxyR is activated by H₂O₂-oxidation and then induces transcription of genes necessary for the bacterial defence against oxidative stress (Choi *et al.* 2001). Other Dps proteins are expressed under conditions of iron starvation in the late log-phase (MrgA_{B. subtilis}, DpsA_{Synechococcus}, non-heme ferritin_{L. innocua}) (Chen *et al.* 1993; Polidoro *et al.* 2002; Sen *et al.* 2000). MrgA from *B. subtilis* is also induced under oxidative stress and the *mrgA* promoter is under the control of PerR. PerR is a member of the Fur family exhibiting two metal binding sites per monomer: one for Zn²⁺ providing structural stability and another one for Fe²⁺ serving as a regulatory element. It is suggested that H₂O₂ reacts with a cysteine involved in iron coordination, leading to a

disulphide bond and eventually releasing iron from its binding site. It is inferred, that the loss of iron in PerR leads to a loss of DNA binding affinity thus enabling transcription (Mongkolsuk & Helmann 2002). In general, *dps* transcription seems to be induced under oxidative stress in exponentially growing cells and/or in the stationary growth phase under iron-limited growth conditions. The transcription is blocked under iron rich growth conditions.

This is in contrast to the Northern-blot results of this study, because the transcription decreases under iron-limited growth and increases under iron rich growth. Moreover, the transcription decreases under iron limited growth and oxidative stress. Therefore, an expression pattern – like that observed for the Dps of *E. coli* – can be excluded. The transcription profile of halobacterial DpsA appears, however, to be the same as for the FtnA/Bfr system in *E. coli* and for Pfr in *H. pylori*.

Based on the data presented here the question arises which transcription factor is responsible for the observed regulation-mode of the *dpsA* gene activity. In an alignment study we were able to show that potential binding sequences for transcription factors DtxR, Fur, OxyR and PerR are present in the upstream region of the halobacterial *dpsA* gene. The highest identity is found for the binding sequence of the DtxR binding protein (68%), followed by the PerR binding sequence (67%) and the Fur binding box from *E. coli* (63%). The binding sequence of OxyR (59%) and the Fur binding box from *H. pylori* (45%), however, display a slightly lower sequence identity.

If OxyR would be the promoter binding protein one would expect an increase of the *dpsA* transcript under conditions of oxidative stress under iron limited growth because OxyR is a sensor solely for oxidative stress. However, this is not the case. In contrast, the Northern blot analysis shows a decrease of the transcription under conditions of iron limited growth and oxidative stress. If PerR would bind at the promoter region one would expect an increase of the transcription under iron limited growth because PerR activates genes when iron is not bound to the protein. This is not the case. The Northern blot analysis displays a decrease of the transcription under these growth conditions. Based on these results we can exclude an OxyR or PerR – like activation of *dpsA* in *H. salinarum*.

We observed decreasing transcription in the Northern blots for both, iron limited growth and iron limited growth under conditions of oxidative stress. Taking into account the error bars of the experimental data we explicate this result as a basal transcription at an integral density of about 0.2%. In contrast to the Northern blot result Western blots show an increasing expression of DpsA concomitant to incubation time and under all growth conditions tested. We attribute this finding to a very long half life of DpsA as it is observed for members of the ferritin superfamily. In this context even a basal transcription leads to

an accumulation of the protein in the cytosol over time.

The increasing transcript abundance observed in the Northern blot analysis under conditions of iron rich growth is consistent with a possible Fur or DtxR protein binding, an observation which is also found in general ferritin regulation. Interestingly, merely a DtxR-homologue has been detected in the halobacterial genome (named *sirR*, 32–36% identity in 224–226 amino acid overlap). In addition, the Northern blot results display a basal transcription under all growth conditions applied in the early logarithmic growth phase and

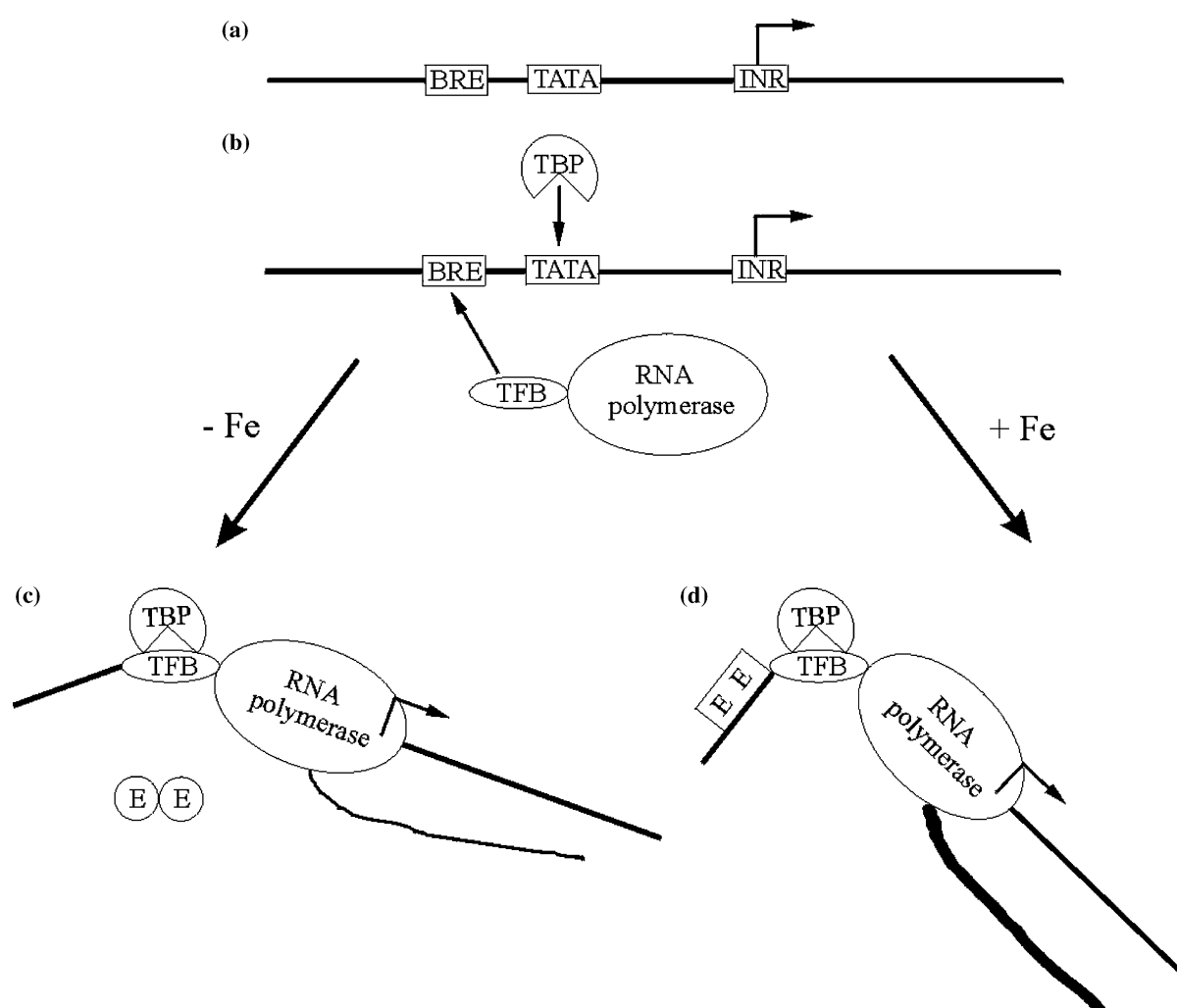


Figure 6. Scheme for the suggested *dpsA* regulation. (a) The archaeal promoter region. (b) Formation of the pre-initiation complex. (c) Suggested transcription under condition of iron deficiency. (d) Suggested transcription under iron rich growth conditions. Abbreviations: BRE (Transcription factor element B), TATA (TATA-box), INR (Initiator), TBP (TATA-box binding protein), TFB (transcription factor B required for a proper positioning of the polymerase), E (effector). BRE, TATA-box and TBP are of primary importance for the promoter specificity.

under conditions of iron limitation over the whole incubation period. We interpret these observations as a result of the basal transcription apparatus in Archaea (Reeve 2003). The increased level of “free” iron in the cytosol under iron rich growth probably activates an hitherto unknown iron-regulated promoter binding protein which enhances ferritin transcription (for a detailed discussion of the term “free” iron see: (Böhnke & Matzanke 1995)). Under conditions of iron rich growth combined with oxidative stress the oxidatively damaged iron–sulfur clusters might release iron into the cytosol as observed in *E. coli* (Imlay 2002). As a consequence, the level of “free” iron is more elevated resulting in a very rapid increase of the transcription under that growth condition. Such an increase of transcription should not occur in the case of iron limited growth under oxidative stress because recent reports indicate that *Halobacterium* replaces iron-containing enzymes and proteins by alternative systems not requiring iron as a co-factor (Hubmacher *et al.* 2003). The suggested regulation process is summarized in a scheme (see Figure 6).

In conclusion, the halobacterial protein DpsA is truly a ferritin. The Northern blot analysis uncovers a regulation pattern which is inverse compared to *dps*. The result from the Western blot analysis reflects a regulation which parallels the few bacterial systems known so far but not the post-transcriptionally regulated eukaryotic ferritins. The latter finding is of special interest because the archaeal transcription system does not *per se* exclude a ferritin regulation typical of eukaryotes. However, the Dps-ferritin of *Halobacterium* appears to operate with regulators typical of bacteria modulating the activity of a basal transcription apparatus like in eukarya. Based on our findings we would suggest to rename *dpsA*/DpsA of *H. salinarum*. An appropriate name would be: Dps homologous dodecameric bacterial ferritin, *ddf*/Ddf.

Acknowledgements

We thank A. Hiller for excellent assistance (immunization of the rabbit) and Britt-Marie Sjöberg for helpful discussions. This work was supported by DFG grant Ma 916/14–1.

References

- Abdul-Tehrani H, Hudson AJ, Chang Y-S, Timms AR, Hawkins C, Williams JM, Harrison PM, Guest JR, Andrews SC. 1999 Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and *fur* mutants are iron deficient. *J Bacteriol* **181**, 1415–1428.
- Almirón 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, Almirón M, Huisman G, Kolter R, Storz G. 1994 The *dps* promoter is activated by OxyR during growth and by IHF and σ^S in stationary phase. *Mol Microbiol* **13**, 265–272.
- Andrews SC. 1998 Iron storage in bacteria. In: Poole RK (Ed.), *Adv Microb Physiol. Vol. 40* London, San Diego: Academic Press, pp. 281–351.
- Azam TA, Hiraga S, Ishihama A. 2000 Two types of localization of the DNA-binding proteins within the *Escherichia coli* nucleoid. *Genes Cells* **5**, 613–626.
- Bereswill S, Greiner S, Van Vliet AHM, Waidner B, Fassbinder F, Schlitz E, Kusters JG, Kist M. 2000 Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. *J Bacteriol* **182**, 5948–5953.
- Böhnke R, Matzanke BF. 1995 The mobile ferrous iron pool in *Escherichia coli* is bound to a phosphorylated sugar derivative. *BioMetals* **8**, 223–230.
- 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.
- Chen L, Helmann JD. 1995 *Bacillus subtilis* MrgA is a Dps (PexB) homologue: evidence for metallorepression of an oxidative-stress gene. *Mol Microbiol* **18**, 295–300.
- Chen L, James LP, Helmann JD. 1993 Metalloregulation in *Bacillus subtilis*: isolation and characterization of two genes differentially repressed by metal ions. *J Bacteriol* **175**, 5428–5437.
- Choi H-J, Kim S-J, Mukhopadhyay P, Cho S, Woo J-R, Storz G, Ryo S-E. 2001 Structural basis of the redox switch in the oxyR transcription factor. *Cell* **105**, 103–113.
- Chomczynski P. 1992 One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal Biochem* **201**, 134–139.
- Chomczynski P, Sacchi N. 1987 Single-step method RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162**, 156–159.
- Delany I, Pacheco ABF, Spohn G, Rappuoli R, Scarlato V. 2001 Iron-dependent transcription of the *frpB* gene of *Helicobacter pylori* is controlled by the fur repressor protein. *J Bacteriol* **183**, 4932–4937.
- Escolar L, Pérez-Martín J, Lorenzo Vde. 1999 Opening the iron box: transcriptional metallorepression by the fur protein. *J Bacteriol* **181**, 6223–6229.
- Evans DJ Jr., Evans DG, Takemura T, Nakano H, Lampert HC, Graham DY, Granger DN, Kvietys PR. 1995 Characterization of a *Helicobacter pylori* neutrophil-activating protein. *Infect Immun* **63**, 2213–2220.
- Fuangthong M, Herbig AF, Bsat N, Helmann JD. 2002 Regulation of *Bacillus subtilis* *fur* and *perR* Genes by PerR: Not All Members of the PerR Regulon Are Peroxide Inducible. *J Bacteriol* **184**, 3276–3286.

- Grant RA, Filman DJ, Finkel SE, Kolter R, Hogle JM. 1998 The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nature Struct Biol* **5**, 294–303.
- Gupta S, Pandit SB, Srinivasan N, Chatterji D. 2002 Proteomics analysis of carbon-starved *Mycobacterium smegmatis*: induction of Dps-Like protein. *Protein Engin* **15**, 503–511.
- Hantke K. 2001 Iron and metal regulation in bacteria. *Curr Opin Microbiol* **4**, 172–177.
- Hubmacher D, Matzanke BF, Anemüller S. 2003 Effects of iron limitation on the respiratory chain and the membrane cytochrome pattern of the euryarchaeon *Halobacterium salinarum*. *Biol Chem* **384**, 1565–1573.
- Ilari A, Stefanini S, Chiancone E, Tsernoglou D. 2000 The dodecameric ferritin from *Listeria innocua* contains a novel intersubunit iron-binding site. *Nature Struct Biol* **7**, 38–43.
- Imlay JA. 2002 How oxygen damages microbes: oxygen tolerance and obligate anaerobiosis. *Adv Microb Physiol* **46**, 111–153.
- Ishikawa T, Mizunoe Y, Kawabata S-i, Takade A, Harada M, Wai SN, Yoshida S-I. 2003 The iron-binding protein Dps confers hydrogen peroxide stress resistance to *Campylobacter jejuni*. *J Bacteriol* **185**, 1010–1017.
- Lanyi JK. 1974 Salt-dependent properties of proteins from extremely halophilic bacteria. *Bacteriol Rev* **38**, 272–290.
- Listowsky I, Blauer G, England S, Bethel JJ. 1972 Denaturation of horse spleen ferritin in aqueous guanidinium chloride solutions. *Biochem* **11**, 2176–2181.
- Massé E, Gottesman S. 2002 A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci USA* **99**, 4620–4625.
- McHugh JP, Rodriguez-Quinones F, Abdul-Tehrani H-Svistunenko DA, Poole RK, Cooper CE, Andrews SC. 2003 Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. *J Biol Chem* **278**, 29478–29486.
- Mongkolsuk S, Helmann JD. 2002 Regulation of inducible peroxide stress response. *Mol Microbiol* **45**, 9–15.
- Ng WV, Kennedy SP, Mahairas GG, Berquist B, Pan M, Shukla HD, Lasky SR, Baliga NS, Thorsson V, Sbrogna J, Swartzell S, Weir D, Hall J, Dahl TA, Welti R, Goo YA, Leithauser B, Keller K, Cruz R, Danson MJ, Hough DW, Maddocks DG, Jablonski PE, Krebs MP, Angevine CM, Dale H, Isenbarger TA, Peck RF, Pohlschroder M, Spudis JL, Jung KW, Alam M, Freitas T, Hou S, Daniels CJ, Dennis PP, Omer AD, Ebhardt H, Lowe TM, Liang P, Riley M, Hood L, DasSarma S. 2000 Genome sequence of *Halobacterium* species NRC-1. *Proc Natl Acad Sci USA* **97**, 12176–12181.
- 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.
- Pena MMO, Bullerjahn GS. 1995a The DpsA protein of *Synechococcus* sp. Strain PCC 7942 is a DNA-binding hemoprotein. *J Biol Chem* **270**, 22478–22482.
- Pena MMO, Burkhart W, Bullerjahn GS. 1995b Purification and characterization of a *Synechococcus* sp. strain PCC 7942 polypeptide structurally similar to the stress-induced Dps/PexB protein of *Escherichia coli*. *Arch Microbiol* **163**, 337–344.
- Polidoro M, Biase DDe, Montagnini B, Guarrera L, Cavallo S, Valenti P, Stefanini S, Chiancone E. 2002 The expression of the dodecameric ferritin in *Listeria* spp. is induced by iron limitation and stationary growth phase. *Gene* **296**, 121–128.
- Poster-Jordan K, Rosenberg EI, Keiser JF, Gross JD, Ross AM, Nasim S, Garrett CT. 1990 Nested polymerase chain reaction assay for the detection of cytomegalovirus overcomes false positives caused by contamination with fragmented DNA. *J Med Virol* **30**, 85–91.
- Pulliaainen AT, Haataja S, Kähkönen S, Finne J. 2003 Molecular basis of H₂O₂ resistance mediated by streptococcal Dps. *J Biol Chem* **278**, 7996–8005.
- Quian Y, Lee JH, Holmes RK. 2002 Identification of a DtxR-regulated operon. That is essential for siderophore-dependent iron uptake in *Corynebacterium diphtheriae*. *J Bacteriol* **184**, 4846–4856.
- Reeve JN. 2003 Archaeal chromatin and transcription. *Mol Microbiol* **48**, 587–598.
- Reindel S, Anemüller S, Sawaryn A, Matzanke BF. 2002 The DpsA-homologue of the archaeon *Halobacterium salinarum* is a ferritin. *Biochim Biophys Acta* **1598**, 140–146.
- Ren B, Tibbelin G, Kajino T, Asami O, Ladenstein R. 2003 The multi-layered structure of Dps with a novel di-nuclear ferroxidase center. *J Mol Biol* **329**, 467–477.
- Sen A, Dwivedi K, Rice KA, Bullerjahn GS. 2000 Growth phase and metal-dependent regulation of the *dpsA* gene in *Synechococcus* sp. strain PCC 7942. *Arch Microbiol* **173**, 352–357.
- Soppa J. 2001 Basal and regulated transcription in archaea. In: Blum P, eds. *Adv Appl Microbiol. Vol. 50* San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, pp. 171–213.
- Sreeramulu K, Schmidt CL, Schäfer G, Anemüller S. 1998 Studies of the electron transport chain of the euryarchaeon *Halobacterium salinarum*: indications for a type II NADH dehydrogenase and a complex III analog. *J Bioenerget Biomem* **30**, 443–453.
- Testa U. 2002 Recent developments in the understanding of iron metabolism. *Hematol J* **3**, 63–89.
- Tindall BJ. 1992 The family halobacteriaceae. In: *The Prokaryotes*, vol. Second edition, Springer Verlag; pp. 768–808.
- 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.
- Torti FM, Torti SV. 2002 Regulation of ferritin genes and protein. *Blood* **99**, 3505–3516.
- Van Vliet AHM, Stoof J, Vlasblom R, Wainwright SA, Hughes NJ, Kelly DJ, Bereswill S, Bijlsma JJE, Hoogenboezem T, Vandenbroucke-Grauls CMJE, Kist M, Kuipers EJ, Kusters JG. 2002 The role of the ferric uptake regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. *Helicobacter* **7**, 237–244.
- Waidner B, Greiner S, Odenbreit S, Kavermann H, Velayudhan J, Stähler F, Guhl J, Bissé E, Vliet AHMVan, Andrews SC, Kusters JG, Kelly DJ, Haas R, Kist M, Bereswill S. 2002 Essential role of ferritin Pfr in *Helicobacter pylori* iron metabolism and gastric colonization. *Infect Immun* **70**, 3923–3929.
- Yamamoto Y, Higuchi M, Poole LB, Kamio Y. 2000 Role of the *dpr* product in oxygen tolerance in *Streptococcus mutans*. *J Bacteriol* **182**, 3740–3747.
- Zanotti G, Papinutto E, Dundon WG, Battistutta R, Seveso M, Del Giudice G, Rappuoli R, Montecucco C. 2002 Structure of the neutrophil-activating protein from *Helicobacter pylori*. *J Mol Biol* **323**, 125–130.