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Ferritin-like family proteins in the anaerobe *Bacteroides fragilis*: when an oxygen storm is coming, take your iron to the shelter

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Abstract

Bacteroides are gram-negative anaerobes and one of the most abundant members the lower GI tract microflora where they play an important role in normal intestinal physiology. Disruption of this commensal relationship has a great impact on human health and disease. *Bacteroides* spp. are significant opportunistic pathogens causing infections when the mucosal barrier integrity is disrupted following predisposing conditions such as GI surgery, perforated or gangrenous appendicitis, perforated ulcer, diverticulitis, trauma and inflammatory bowel diseases. *B. fragilis* accounts for 60–90 % of all anaerobic infections despite being a minor component of the genus (<1 % of the flora). Clinical strains of *B. fragilis* are among the most aerotolerant anaerobes. When shifted from anaerobic to aerobic conditions *B. fragilis* responds to oxidative stress by inducing the expression of an extensive set of genes involved in protection against oxygen derived radicals and iron homeostasis. In *Bacteroides*, little is known about the metal/oxidative stress interactions and the mobilization of intra-cellular non-heme iron during the oxidative stress response has been largely overlooked. Here we present an overview of the work carried out to demonstrate that both oxygen-detoxifying enzymes and iron-storage proteins are essential for *B. fragilis* to survive an adverse oxygen-rich environment. Some species of *Bacteroides* have acquired multiple homologues of the iron storage and detoxifying ferritin-like proteins but some species contain none. The proteins found in *Bacteroides* are classical mammalian H-type non-heme ferritin (FtnA), non-specific DNA binding and starvation protein (Dps) and the newly characterized bacterial Dps-Like miniferritin protein. The full contribution of ferritin-like proteins to pathophysiology of commensal and opportunistic pathogen *Bacteroides* spp. still remains to be elucidated.

Keywords

Bacteroides fragilis ferritin superfamily; Iron-storage and detoxification in anaerobes; Ferritin; Dps; Dps-Like

Introduction

The human colon is the most densely populated organ with commensal microbes. The low level of oxygen and low redox potential creates an optimal anaerobic environment where obligate anaerobes are the prevalent microorganisms. Among these anaerobes, *Bacteroides* species are predominant members of the human gut normal microbiota. Colonization of the intestinal tract by *Bacteroides* spp. is fundamental for the establishment and maintenance of a normal, healthy intestinal microbiota and disruption of this commensal relationship has a great impact on health and disease. In the human colon, *Bacteroides* spp. can reach numbers in excess of 10^{11} cells per gram of content and account for about 30–40 % of total bacteria where at least 500–1,000 different species have been so far reported (Savage 1977; Gibson and Roberfroid 1999; Hooper et al. 2002; Eckburg et al. 2005; Smith et al. 2006; Reading and Kasper 2011). In the lower intestinal tract, *Bacteroides* spp. contribute to several beneficial activities such as complex polysaccharide degradation, protection of the gut epithelia from colonization by pathogenic bacteria, development of the intestinal tract, maturation of the mucosal and systemic immune systems, bile acid turnover metabolism, energy harvesting, proteolytic activity and transformation of toxic and mutagenic compounds (Bernalier et al. 1999; Gibson and Roberfroid 1999; Hooper et al. 2002; Bäckhed et al. 2004; Eckburg et al. 2005; Smith et al. 2006; Neu et al. 2007; Tappenden and Deutsch 2007; Turnbaugh et al. 2006; Wexler 2007; Neish 2009; Sekirov et al. 2010; Reading and Kasper 2011).

There are over 20 cultivable species of the genus *Bacteroides* of which *Bacteroides vulgatus*, *B. thetaiotaomicron* and *Parabacteroides distasonis* are the most abundant species in the human colon (Wexler 2007). Though this group of bacteria forms a very close and distinct cluster within the *Bacteroidetes* (Cytophaga-Flavobacteria-Bacteroides) phylum based on their 16S rRNA homology sequences, it has been established that they share little overall DNA identity. As an example, the type strain *B. fragilis* only shares 5–10 % DNA identity with *B. vulgatus*, 13–22 % with *B. ovatus* and 21–36 % with *B. thetaiotaomicron*. This demonstrates the high nucleotide sequence heterogeneity among the species but despite this they share common features such as host association and colonization of anaerobic niches, and they are relatively uniform with regard to their metabolic, genetic and physiological properties (Smith et al. 2006).

Opportunistic infections caused by *Bacteroides* spp. occur as a consequence of a disruption in the integrity of the mucosa wall where intestinal luminal content escapes into the peritoneal cavity. Secondary peritonitis due to bacterial contamination of the peritoneal cavity is the result of conditions such as GI tract surgery, perforated or gangrenous appendicitis, perforated ulcer, diverticulitis, trauma, perforated colon cancer and inflammatory bowel diseases (McClean et al. 1994; van Till et al. 2007; Mazuski and Solomkin 2009). While most of the contaminant bacteria will be cleared by the host immune defenses within minutes, *B. fragilis*, which comprises only 0.5–1 % of the human normal intestinal microflora, emerges as the most frequent anaerobe isolated from intra-abdominal abscesses, peritonitis, infections of the female genital tract, deep wounds, brain abscesses and bacteremia. *B. fragilis* accounts for about 50–70 % of all anaerobic bacteria isolated from human infections (Brook 1989; Finegold and George 1989; McClean et al. 1994; Brook and Frazier 2000; Mazuski and Solomkin 2009; Park et al. 2009). The pathogenicity traits of *B. fragilis* are not completely understood but virulence factors such as capsular polysaccharides, adherence, production of proteases, neuraminidase, iron acquisition and resistance to oxidative stress play an important role (Smith et al. 2006; Wexler 2007).

Very little is known about the oxidative stress response in *Bacteroides* and nearly nothing has been published on the mechanisms they employ to manage the intracellular iron pool. In

bacteria, the ferritin-like family; the classical non-heme mammalian H-type ferritin (FtnA), the heme-containing bacterioferritin (Bfr), the DNA-binding and starvation protein (Dps) (Andrews 2010) and the novel archaeal and bacterial Dps-Like thioferritins (Gauss et al. 2006, 2012) is a distinct group of proteins whose function is to mobilize and store iron using the protein's spherical hollow shell as a means to limit iron availability to control oxidative stress damage. Though they share 3-dimensional structure properties, they differ in primary amino acid sequence. Ferritin and bacterioferritin limit the availability of free Fe^{++} by utilizing oxygen for oxidation of Fe^{++} into an insoluble iron core within the 24-meric spherical shell (Andrews 2010). The dodecameric DNA-binding and stationary-phase protection protein, Dps, sequesters Fe^{++} by using H_2O_2 for iron oxidation without producing hydroxyl radicals (Zhao et al. 2002; Calhoun and Kwon 2010). It is not our purpose in this paper to review the details of structure, regulation and functional properties of the ferritin-like superfamily. For this, a wide-range of excellent publications are available (Andrews 1998, 2010; Bou-Abdallah 2010; Calhoun and Kwon 2010). Our purpose in this short review is to present an overview of our current knowledge on the role of the ferritin-like family of iron-storage proteins in *B. fragilis* oxidative stress resistance. We will focus mostly on *B. fragilis* because this has been the only model organism investigated thus far while some *Bacteroides* species will be addressed for comparison purposes.

Oxidative stress response overview

Our major interest in *B. fragilis* comes from early studies demonstrating that clinical isolates of *B. fragilis* are by far the most aerotolerant anaerobic bacteria and survive 2–3 days in the presence of atmospheric oxygen while non-clinical isolates lose viability within hours (Rolfe et al. 1977; Tally et al. 1975). This remarkable aerotolerance that enables clinical isolates to survive in oxygenated tissues during the initial stages of infection in extra-intestinal infections is linked to their inducible aerotolerance properties (Rocha et al. 2007; Sund et al. 2008). Thus, when *B. fragilis* is shifted from the intestinal anaerobic environment to aerobic conditions in oxygenated extra-intestinal tissues, it must regulate its energy resources and metabolism to protect against oxidative stress and the oxidative burst from immune phagocytic defenses.

When *B. fragilis* 638R is exposed to atmospheric oxygen, a clear picture of genetic and physiological complexity of its response has started to emerge. When bacteria are exposed to oxygen, the reactive oxygen species, hydrogen peroxide and superoxide anion, will be generated. This can be a serious problem because soluble ferrous iron will readily react with hydrogen peroxide forming the toxic hydroxyl radical through the Fenton reaction ($\text{Fe}^{++} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^\bullet + \text{OH}^- + \text{Fe}^{+++}$). Consequently, to avoid the generation of toxic cell-damaging oxygen radicals, bacteria have developed detoxifying enzymes such as superoxide dismutase to dismutate superoxide anion into H_2O_2 , and catalases and peroxidases to eliminate H_2O_2 . To maintain intra-cellular iron at non-toxic levels, bacteria mobilize free iron into iron-storage and detoxifying proteins in a non-reactive insoluble ferric form (Andrews 1998; Touati 2000). When mid-log growth cultures of *B. fragilis* were exposed to oxygen or treated with sublethal concentration of H_2O_2 , they induced the synthesis of at least 28 and 23 proteins respectively following 2D SDS-PAGE analysis (Rocha et al. 1996). This response was further analyzed by measuring the whole transcriptional response to oxidative stress and the results revealed an immense change in gene expression that affects 45 % of the genome (Sund et al. 2008). The greatest effect on gene expression was seen in cultures air exposed in which 396 genes were induced and 368 were repressed (Sund et al. 2008). This oxidative stress response is necessary for *B. fragilis* to survive in the presence of atmospheric oxygen and some of these inducible genes have been demonstrated to be important for survival in intra-intestinal infections (Rocha et al. 2007; Sund et al. 2008). Among these oxidative stress response genes, the ferritin-like family protein members

present in *B. fragilis*, (FtnA, Dps and the bacterial Dps-Like [formerly bacterioferritin *bfi*-related gene] proteins) are significantly induced by iron and oxidative stress in northern blot and microarray expression studies (Rocha et al. 2000; Rocha and Smith 2004; Sund et al. 2008). For this reason, we have investigated the roles of the FtnA, Dps-Like and Dps proteins in *B. fragilis* oxidative stress resistance.

Ferritin (FtnA)

The presence of FtnA in *B. fragilis* is of considerable interest because it demonstrates that storage of excess iron in the *Bacteroides* group may be an important strategy developed to prevent the production of toxic free radicals through the Fenton reaction when bacteria are shifted from an anaerobic to an aerobic environment (Rocha and Smith 2004; Sund et al. 2008). The importance of iron storage in *Bacteroides* is illustrated by the fact that many species of *Bacteroides* carry multiple *ftnA* genes. For example, the most abundant *Bacteroides* species isolated from fecal samples, *B. thetaiotaomicron* and *B. vulgatus* (Hooper et al. 2002) possess 3 FtnA homologues respectively in their genomes while other less abundant species contain one or no homologues (Table 1). A comparison of the genetic organization of FtnA1 (numbered in reference to *B. fragilis* FtnA) among *Bacteroides* is shown in Fig. 1. The upstream chromosomal region is well conserved among major representative species of the genus except for *B. vulgatus* ATCC 8482. There are no conserved loci arrangements flanking downstream region the *ftnA1*. *B. fragilis* 638R FtnA shares 100 % amino acid identity with *B. fragilis* NCTC 9343 and YCH46 and ~ 89-55 % identity to FtnA1 and FtnA2 from other *Bacteroides* species. FtnA1 shows <29 % identity to FtnA3 homologues.

An alignment of the *Bacteroides* and *Parabacteroides* FtnA protein homologues showed that they contain the conserved di-nuclear iron sites of the ferroxidase center, except for the FtnA2 of *B. dorei*, *B. thetaiotaomicron*, *B. vulgatus*, *P. merdae* and *P. plebeius* where Asp129 substitutes Glu129 (Fig. 2). Among the FtnAs, the *B. fragilis* FtnA and *P. distasonis* FtnA1 are the only ones that do not contain conserved cysteine residues. It remains to be investigated whether cysteine residues in the FtnAs of anaerobic bacteria play a role in ferritin oxidation, iron loading, and formation of ferritin aggregates (Welch et al. 2002). Analysis of a phylogenetic tree constructed from multiple aligned amino acid sequences using the maximum likelihood method showed that FtnA3 homologues are distinctly clustered in separate branch from the FtnA1 and FtnA2 groups (Fig. 3). Phylogenetically, FtnA1 and FtnA2 are related to archaea ferritins in a separate cluster from other prokaryotes while FtnA3 is related to Mycobacteria and eukaryotes (Rocha and Smith 2010). Moreover, a comparison of the genetic organization of FtnA1, FtnA2, FtnA3, DpsL, and Dps homologues in *Bacteroides* and *Parabacteroides* species with complete ungapped circular chromosomes, revealed that they are found at different locations relative to the chromosome origin of replication *oriC* (Fig. 4). This suggests that there have been major genetic rearrangements among these species.

In *Bacteroides* species containing multiple homologues of FtnA such as in *B. thetaiotaomicron*, *B. vulgatus*, *B. uniformis* and *B. dorei*, FtnA2 and FtnA3 are organized in a putative polycistronic operon of approximate 12 kb (Fig. 5). It is quite unusual for bacteria to carry an extended number of ferritins such as in *Bacteroides* species since most bacteria carry two ferritins, two bacterioferritins or one of each and some lack any known ferritin family of iron storage protein (Andrews 1998). This unusual characteristic seems to be uniquely associated with host-associated intestinal *Bacteroides* strains as free-living members of the *Bacteroidetes* phylum such as *Cytophaga* and *Flavobacterium* contain no more than one or two ferritin family homologues (not shown). The *ftnA2* and *ftnA3* genes are clustered together on the genome with (1) *marC* (gene of unknown function, previously

annotated as multiple antibiotic resistance gene, McDermott et al. 2008), (2) genes involved in energy central metabolism [class I fructose-1,6-bisphosphate aldolase (FbaB), phosphoglyceromutase (GpmA), 6-phosphofructokinase (PfkA), alpha-glycan phosphorylase (GT1), methylglyoxal synthase (MgsA), Sodium/proton antiporter (TrkA)] (3) stress response [small heat shock protein, (HSP20), ECF-type sigma factor, and (4) amino acid metabolism [Arginase/agmatinase/formimionoglutamate hydrolase (SpeB)]. The FtnA3 of *B. vulgatus* ATCC 8482 and *B. dorei* DSM 17855 contain only a partial ferritin primary structure with 100 and 99 % identity, respective, to the N-terminus and C-terminus domains of FtnA3 from *B. thetaiotaomicron* and *B. uniformis*. Unfortunately, there are no reports on the regulation and role of the extended number of ferritins in some *Bacteroides* species but they may be required for rigorous control of their iron pools to protect against ROS damage or they may provide a competitive nutritional advantage to scavenge and store a higher amount of iron than other bacteria.

In *B. fragilis*, the *ftnA* gene is expressed as a monocistronic mRNA and the basal level of expression under anaerobic conditions is not altered by high- or low-iron concentrations in vitro. This is consistent with the fact that under anaerobic conditions, ferrous iron imposes no toxicity to the cell and detoxification seems to be unnecessary. However, when conditions change to an oxygenated environment, *ftnA* mRNA increases over 10-fold in iron-replete medium but in low-iron medium, the presence of oxygen induces *ftnA* expression to a modest 4-fold (Rocha and Smith 2004). In the presence of the oxidants diamide (a thiol oxidant that mimics the effect of oxygen on anaerobes) and potassium ferricyanide, *B. fragilis* *ftnA* expression is upregulated 5–12-fold. The control of oxidative stress response genes in *B. fragilis* is tightly regulated and the simultaneous coordinate regulation of *B. fragilis* *ftnA* expression by iron and oxygen indicates that the management of intracellular iron and oxidative stress response are intrinsically associated to minimize or avoid the cellular oxidative damage. One of the ROS response regulatory mechanisms characterized in *B. fragilis* is the peroxide response regulator OxyR (Rocha et al. 2000; Rocha and Smith 2004; Sund et al. 2008). After oxidative stress, OxyR rapidly induces the expression of several genes including *ftnA*, *dps* and the *dps*-like genes (discussed below) (Rocha and Smith 2004; Sund et al. 2008; Gauss et al. 2012). These studies have demonstrated that the ferritin-like family proteins also have an OxyR-independent oxygen-dependent regulatory mechanism that has not yet been characterized. Evidence that oxidative stress and intracellular iron mobilization are intrinsically connected with oxidative damage in *B. fragilis* comes from mutational studies showing that *ftnA*, *dps* or *oxyR* mutants are no more sensitive to oxygen exposure than the wild-type during extended exposure to atmospheric air. However, when these mutations were combined such as in the *dps oxyR* and *ftnA dps oxyR* strains, significant survival defects occurred compared to the single mutants (Rocha and Smith 2004).

Dps-Like thioferritin

Until recently, a bacterioferritin-related homologue (*bfi*) gene had been annotated in *B. fragilis* 638R. However, structural analysis demonstrated it is distinct from both the larger Ftn and Bfr 24-mers and from the dodecameric Dps. It is more closely related to archaeal *Sulfolobus solfataricus* Dps-Like protein. Like archaeal DpsL, Bf-Bfr (referred herein as Bf-DpsL) is distinguished by a pair of conserved cysteine residues, has a unique third metal site, has a different conformation of the extended C-terminus D-helix and the number and locations of the major pores (Gauss et al. 2012). Like Dps, archaeal DpsL and Bf-DpsL are expressed in response to oxygen.

Among the *Bacteroidetes* phylum, Bf-Dps-Like mini-thioferritin seems to be restricted to host-associated species that colonizes the low intestinal tract belonging to the genus:

Bacteroides, *Parabacteroides*, *Oridobacter* and *Barnesiella*. Also, Bf-DpsL homologues are not present in the free-living Cytophaga-Flexibacter-Flavobacteria members of the *Bacteroidetes* (not shown). This indicates that they might be adapted to deal with iron mobilization/storage in diverse ecological habitats. The DpsL genetic locus in *B. fragilis* 638R does not share conserved chromosomal organization to other *Bacteroides* species (Fig. 6). In addition to the genetic variability and rearrangements of *dpsL* chromosomal location (Fig. 4), the Bf-dpsL homologue is not widely distributed among the *Bacteroides* and *Parabacteroides* strains (Table 1). Less than 50 % (10/25) of the strains listed in Table 1 contain one homologue of DpsL while the majority of the strains have none. The di-iron metal binding sites (Glu29, Glu62, His65, Glu114, Glu146, and His149) and the cysteine residues (Cys93 and Cys116) of bacterial and archaeal mini-thioferritin Dps-Like proteins (Gauss et al. 2012) are highly conserved in the *Bacteroides* DpsL homologues (Fig. 2). The third metal binding site (Glu54, His58, and Asp152) present in the bacterial type of DpsL homologues is also conserved.

Bf-*dpsL* mRNA is induced approximately 35-fold by oxygen exposure and this induction has a further 15-fold increase in the presence of excess iron but there is no significant alteration of Bf-*dpsL* mRNA by excess iron in anaerobic culture controls (Gauss et al. 2012). A post-transcriptional regulation related to growth-phase conditions seems to limit the protein expression as there was only an ~1.8-fold and 3.8-fold increase in the amount of DpsL under aerobic conditions and stationary phase cultures compared to anaerobic culture controls. Despite notable increase of Bf-*dpsL* mRNA in the presence of oxygen, no transcription increase is observed during H₂O₂ exposure in a microarray study (Sund et al. 2008). This may be a consequence of the fact that in the wild type strain a rapid response of the OxyR regulated catalase and AhpCF can hastily eliminate H₂O₂ preventing induction of Bf-*dpsL* because there is a 4-fold induction of Bf-*dpsL* in the *oxyR* mutant strain with a drastic reduction in the *katB* and *aphCF* expression. Consistent with the participation of OxyR in *ftnA* regulation, Bf-*dpsL* also exhibits coordinated regulation by both iron and oxygen with an OxyR-dependent and OxyR-independent oxygen-dependent regulatory model (Sund et al. 2008; Gauss et al. 2012).

From mutation studies a *dpsL* and a *dpsL ftnA* mutant showed increased sensitivity to cumene hydroperoxide while sensitivity to diamide is only observed in the double *dpsL ftnA* mutant. But neither single mutants nor double mutant showed sensitivity to H₂O₂ in disk inhibition assays (Gauss et al. 2012). When these strains were exposed to prolonged aerobic survival experiments, the wild type strain survived beyond 84 h, while the *dpsL* and *ftnA* single mutants lost viability by 2-orders of magnitude by 60 h of aerobic exposure. The double mutant had a more pronounced loss of viability than the single mutants showing that the roles of the ferritin family proteins in *B. fragilis* involve an elaborate interlinked systems of oxidative stress protection. Studies on the transcriptional expression and protein accumulation showed no evidence for compensation of *ftnA* mutation by overproducing DpsL and vice versa (Gauss et al. 2012). The difference in their regulation and phenotypes may be designed to modulate physiological activities in accord with the levels of iron concentration and oxidative stress conditions.

Dps

The DNA binding protein from starved cells, Dps, was first identified in *B. fragilis* from a highly peroxide resistant strain constitutively expressing KatB, AhpC and Dps (Rocha and Smith 1998). The *dps* gene is expressed as a single monocistronic mRNA divergently transcribed from the transcriptional regulator OxyR. As seen in other bacteria, Bf-OxyR also upregulates the expression of *dps* following treatment with sublethal concentration of H₂O₂. Bf-*dps* is induced nearly 500-fold by 50 µM H₂O₂ or oxygen exposure in Northern blot

analysis (Rocha et al. 2000). Following the same trend in different experiments, *dps* levels were increased 25-fold by H₂O₂ treatment and 38-fold by oxygen exposure in a microarray transcriptome analysis (Sund et al. 2008). OxyR is the major regulator of *dps* as induction of *dps* transcripts was nearly abolished in the *oxyR* mutant treated with H₂O₂ but *dps* was still upregulated 28-fold during oxygen exposure by a regulator that is yet unknown (Sund et al. 2008). This regulatory model is somewhat consistent with all three of the ferritin-like family proteins in *B. fragilis*, i.e., an OxyR-dependent and an OxyR-independent regulatory mechanism. This implies that the ferritin-like family in *B. fragilis* will work in one accord to restrict and manage the intracellular iron pool during an oxidative stress to protect against oxidative damage.

The di-iron site ligands of *E. coli* Dps that coordinate the intersubunit metal binding site (Andrews 2010) are also conserved in *Bacteroides* and *Parabacteroides* Dps homologous (Fig. 2). The Dps proteins found in *B. cellulosilyticus*, *B. eggerthii*, *B. intestinalis*, *B. stercoris*, *B. uniformis*, *P. merdae*, and *P. distasonis* contain two conserved cysteine residues (Cys100 and Cys160). Interestingly, *Porphyromonas gingivalis* Dps contains a single cysteine residue (Cys101, the corresponding residue of *Bacteroides* Dps Cys100) that coordinates a novel hemeiron binding property at the surface of the dodecameric protein (Gao et al. 2012). Phylogenetically, the *Bacteroides* Dps is separated from the FtnA and DpsL branch. The Dps homologous lacking cysteine residues are clustered together in a branch separated from the Dps containing conserved cysteine residues (Fig. 3). It is likely that there might have significant differences in the structures and functions of *Bacteroides* Dps proteins containing cysteine and those lacking cysteine residues. The physiological significance of these differences remains to be determined in view of the fact that they are not widely distributed among the species of the genus (Table 1).

Mutational studies have shown that *dps* and *oxyR* mutants are highly sensitive to H₂O₂ but their viability is not significantly altered by prolonged oxygen exposure. Nonetheless, as mentioned above, when they are putting in combination with *ftnA*, there are a significant reduction in survival compared to single mutants (Rocha and Smith 2004). The importance of *dps* was further demonstrated by its role during the initiation and formation of abscess in a mouse model. The *dps* and *oxyR* single mutants were significantly impaired in the induction of abscess than the wild type. This abscess formation deficiency was even greater in the *dps oxyR* double mutant compared to the wild type strain (Sund et al. 2008).

Perspectives and future directions

There has been great progress in understanding the role, function, and regulation of Ferritin-like super-family proteins in the last few decades. These studies have directed their attention to aerobic organisms likely because aerobic life has an intrinsic metabolic connection between iron and oxygen. For obligate anaerobes where the presence of ferrous iron in the absence of oxygen imposes no toxicity, it becomes quite an intriguing fact that this group of organisms has developed a mechanism to eliminate and detoxify iron in response to oxygen using mechanisms nearly identical to aerobic organisms (Fig. 7). In fact, the *Bacteroides* group, which is phylogenetically divergent from other eubacteria, seems to have a great requirement for controlling intra-cellular iron pool as evidenced by the number of ferritin genes present in their genomes. The gene clusters in bacterial chromosome, usually are arranged according to their related functions and expression control. Thus, we think that future investigations may provide new information on the role and regulation of multiple ferritins in obligate anaerobes. Perhaps, the assumption is wrong that soluble ferrous in anaerobic bacteria is “free” iron in the cytoplasm and that it may not be as free as we think. In fact, we know very little about the iron requirement and storage in *Bacteroides* during intestinal colonization. Further studies may determine whether *Bacteroides* ferritins

contribute in scavenging luminal intestinal iron as nearly 99 % of the total fecal iron is present in the insoluble fecal content in non-reactive form and only about 1.3 % is found in the water soluble fraction in a reactive iron form (Lund et al. 1999).

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Fig. 1.

The genetic structure of the *ftnA1* locus in the genomes of representative *Bacteroides* species. Strain designations are shown in each panel. Bf *B. fragilis*, Bt *B. thetaiotaomicron*, Bo *B. ovatus*, Bu *B. uniformis* and Bv *B. vulgatus*. The chromosomal nucleotide sequences flanking the *ftnA1* genes were retrieved from public genome database (<http://www.ncbi.nlm.nih.gov/>) and used to create a DNA molecule map in Vector NTI Advance 11.5.2. Open reading frame (ORF) features were added to the molecule using the nucleotides position corresponding to the annotated protein locus_tag. The *ftnA1* ORF and direction of transcription is depicted by a *dark arrow*. *Open arrows* depict ORFs and transcription orientation of the genes flanking *ftnA1* chromosomal region. The locus_tag for each gene is shown below its respective ORF and gene assignment. AAT: aspartate aminotransferase superfamily; AK: aspartokinase III; *ftsE*: cell division ATP-binding protein; *hisIE*: histidine biosynthesis bifunctional protein; HK: Histidine kinase-like ATPases; HP: conserved hypothetical protein; *lysA*: diaminopimelate decarboxylase; *nadB*: NAD dependent nucleotide-diphosphate-sugar epimerase; OPT: oligopeptide transporter; S16: 30S ribosomal protein S16; *tnp*: transposase; *xyl*: xylanase

Fig. 2.

Multiple alignments of the *Bacteroides* and *Parabacteroides* deduced amino acid sequences for FtnA, Dps, and DpsL protein homologues. The *E. coli* FtnA and Dps proteins were included for comparison purposes. Conserved amino acid residues (>50 % identity) are labeled with *blue boxes*. Semi-conserved amino acid substitutions are depicted by *green boxes*. Key ferroxidase center residues of FtnA and Dps (Andrews 1998, 2010) are depicted by *one letter*, respectively, below and above the sequence. For DpsL, the di-iron site ligands (Gauss et al. 2012) are depicted by circled one-letter code amino acid below the sequence. The third metal ligand sites of DpsL proteins are depicted by *grey boxes*. The conserved residues of Dps metal ligands motifs are not highlighted. Cysteine residues are labeled in *yellow boxes*. Alignment of protein sequences was performed using the AlignX program component of Vector NTI Advance 11.5.2 with the peptide scoring matrix default data file blosum62mt2 for the comparison of amino acids substitution

Fig. 3.

Phylogenetic analysis of *Bacteroides* and *Parabacteroides* ferritin family of iron-storage proteins. The amino acid sequences of 57 FtnA, Dps and DpsL proteins were aligned using Multiple Sequence Comparison by Log- Expectation (MUSCLE). The tree was constructed in MEGA5 by the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992; Tamura et al. 2011). The *E. coli* FtnA, *E. coli* Dps, and *S. sulfataricus* DpsL proteins were included for comparison purposes. Branches corresponding to partitions reproduced in <50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (50 replicates) are shown next to the branches (Felsenstein 1985). Initial trees for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. All positions containing gaps were eliminated. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Asterisks depict the Dps protein homologous containing conserved cysteine residues

Fig. 4.

Circular map showing the chromosomal locations of FtnA, Dps, and DpsL proteins in *Bacteroides* and *Parabacteroides* species. The protein sequences of *B. fragilis* 638R FtnA (BF638R_2891), Dps (BF638R_1333), and DpsL (BF638R_3305) were, respectively, used to identify the homologous proteins and respective locus_tag number in complete ungapped genomes of *B. thetaiotaomicron* VPI 5482 (NC_004663), *B. vulgatus* ATCC 8482 (CP000139), and *P. distasonis* ATCC 8503 (CP000140). The genomic nucleotide sequences were used to create circular maps in Vector NTI Advance 11.5.2. The gene locus positions were inserted in the map using respective annotated nucleotide sequence positions for each locus_tag. The genome length is depicted in each *panel* below the respective strain name

Fig. 5.

The genetic structure of the *ftnA2* and *ftnA3* loci in the genomes of *Bacteroides* species. Strain designations are shown in each panel. Bt *B. thetaiotaomicron*, Bu *B. uniformis*, Bv *B. vulgatus* and Bd *B. dorei*. The chromosomal nucleotide sequence flanking the *ftnA2* gene was retrieved from public genome database (<http://www.ncbi.nlm.nih.gov/>) and used to create a DNA molecule map in Vector NTI Advance 11.5.2. Open reading frame (ORF) features were added to the molecule using the nucleotides position corresponding to the annotated protein locus_tag. The *ftnA2* ORF and direction of transcription is depicted by a dark grey arrow while *ftnA3* is depicted by silver grey arrows respectively. Open arrows depict ORFs and transcription orientation of the genes flanking *ftnA2* and *ftnA3* chromosomal region. The locus_tag for each gene is shown below its respective ORF and gene assignment. ECF: RNA polymerase ECF-type sigma factor; *fbaB*: fructose-bisphosphate aldolase; *glgA*: alpha-glucan phosphorylase; *gpmA*: phosphoglyceromutase; sHSP: small heat shock protein; *marC*: hypothetical protein; *mgsA*: methylglyoxal synthase; *pfkA*: 6-phosphofructokinase; *speB*: arginase; *trkA*: sodium/proton antiporter

Fig. 6.

The genetic structure of the *dpsL* (formerly *bfr*-related gene) locus in the genomes of representative *Bacteroides* species. Strain designations are shown in each panel. Bf *B. fragilis*, Bt *B. thetaiotaomicron*, Bo *B. ovatus*, Bv *B. vulgatus* and Bd *B. dorei*. The chromosomal nucleotide sequence flanking the *dpsL* gene was retrieved from public genome database (<http://www.ncbi.nlm.nih.gov/>) and used to create a DNA molecule map in Vector NTI Advance 11.5.2. Open reading frame (ORF) features were added to the molecule using the nucleotides position corresponding to the annotated protein locus_tag. The *dpsL* ORF and direction of transcription is depicted by a dark grey arrow. Open arrows depict ORFs and transcription orientation of the genes flanking *dpsL* chromosomal region. The locus_tag for each gene is shown below respective ORF and gene assignment. *acrR*: transcriptional regulator TetR; *crp*: transcriptional regulator; *dagK*: lipid kinase; *ftsW*: rod shape-determining protein; *gapA*: glyceraldehyde 3-phosphate dehydrogenase; *gldH*: gliding motility-associated lipoprotein; *glnA*: glutamine synthetase; *guaA*: GMP synthase; *holB*: DNA polymerase III, delta subunit; HP: conserved hypothetical protein; *kdsA*: 2-dehydro-3-deoxyphosphooctonate aldolase; *lpxK*: tetraacyldisaccharide kinase; *miaA*: tRNA delta(2)-isopentenylpyrophosphate; *metF*: 5,10-methylenetetrahydrofolate reductase; *mscL*: large-conductance mechanosensitive channel; OEP: outer membrane efflux protein; *pbp2_mrdA*: penicillin-binding protein 2; *pnp*: purine nucleoside phosphorylase I; *sppA*: protease IV; *thiL*: thiamine monophosphate kinase; TM: putative transmembrane protein; *tnp*: transposase

Fig. 7.

Schematic model of the interplay between oxidative stress response and intra-cellular iron mobilization in *B. fragilis*. Intra-cellular superoxide anions are predominantly generated from fumarate reductase complex (Frd) and membrane oxidases when *B. fragilis* is exposed to oxygen. Superoxide anions attack iron-sulfur clusters, cause disassociation and release of ferrous iron. Superoxide dismutase eliminates superoxide anions by dismutation with generation of H_2O_2 . Increased intra-cellular levels of H_2O_2 activate the peroxide response regulator OxyR which induces the expression of the peroxide detoxifying enzymes, catalase (KatB), alkyl hydroperoxidase (AhpCF), thioredoxin peroxidase (Tpx) and rubrerythrin (Rbr1). Induction of FtnA, Dps, and DpsL expression is controlled in part by an OxyR-dependent and an unidentified OxyR-independent oxygen-dependent mechanism. Ferrous iron may enter the cell through a putative ferrous iron transporter (FeoAB). Heme is transported into the cell is also a source of ferrous iron when iron is removed from heme with release of intact protoporphyrin IX (PpIX). The enzyme(s) involved in *Bacteroides* heme demetalase activity is yet to be identified. Heme transport systems and ferrous iron transport in *B. fragilis* are empirically represented in the diagram based on putative homologues in its chromosome. The mobilization of free ferrous iron inside anaerobic bacteria cells is not known but it is assumed to be mobilized and loaded into the iron-storage proteins in a non-toxic ferric form following oxidative stress. These mechanisms will prevent the reaction of H_2O_2 with ferrous iron (Fenton reaction) and avoid the formation of toxic hydroxyl radicals ($\text{HO}^\bullet$). Model compiled and adapted from; Rocha et al. (2000, 2003), Imlay (2002), Rocha and Smith (2004, 2010), Meehan and Malamy (2012)

Table 1
Distribution of ferritin-like family genes *fna*, *dpsL* and *dps* in the genomes of representative *Bacteroides* and *Parabacteroides* strains

Strains	<i>fna</i> genes	GenBank accession #	<i>dpsL</i> genes	GenBank accession #	<i>dps</i> genes	GenBank accession #
<i>B. vulgatus</i> ATCC 8482	3	ABR41141 ABR40404 ABR41143* ABR41144*	1	ABR41194	None	–
<i>B. dorei</i> DSM 17855	3	EEB23251 EEB23646 EEB23643* EEB23644*	1	EEB26073	1	EEB25787
<i>B. thetaiotaomicron</i> VPI 5482	3	AAO76480 AAO76214 AAO76216	1	AAO78928	1	AAO79820
<i>B. uniformis</i> ATCC 8492	3	ED051842 ED055572 ED055570	None	–	1	ED055385
<i>B. stercoris</i> ATCC 43183	3	EDS16588 EDS15052 EDS15050	None	–	1	EDS 14745
<i>P. merdae</i> ATCC 43184	3	EDN87770 EDN84345 EDN84347	None	–	1	EDN86811
<i>B. plebeius</i> DSM 17135	3	EDY97050 EDY93787 EDY93785	None	–	None	–
<i>P. distasonis</i> ATCC 8503	2	ABR41895 ABR42042	1	ABR43121	2	ABR42394 ABR42396
<i>B. fragilis</i> NCTC 9343	1	CAH08578	1	CAH08967	1	CAH07034
<i>B. fragilis</i> YCH46	1	BAD49794	1	BAD50193	1	BAD48082
<i>B. fragilis</i> 638R	1	AAK29742	1	CBW23774	1	AAG02618

Strains	<i>finA</i> genes	GenBank accession #	<i>dpsL</i> genes	GenBank accession #	<i>dps</i> genes	GenBank accession #
<i>B. caccae</i> ATCC 43185	1	EDM20344	1	EDM19603	1	EDM20674
<i>B. ovatus</i> ATCC 8483	1	EDO14102	1	EDO11031	1	EDO09702
<i>B. intestinalis</i> DSM17393	1	EDV04331	None	–	1	EDV06219
<i>B. eggerthii</i> ATCC 27754	1	EEC53921	None	–	1	EEC52393
<i>B. cellulosilyticus</i> DSM14838	1	EEF91989	None	–	1	EEF90647
<i>B. coprosuis</i> DSM 18011	1	EGJ71216	None	–	1	EGJ72116
<i>B. coprocola</i> DSM 17136	1	EDV02320	None	–	None	–
<i>B. coprophilus</i> DSM 18228	1	EEF74650	None	–	None	–
<i>B. ureolyticus</i>	None	–	None	–	None	–
<i>B. pyogenes</i>	None	–	None	–	None	–
<i>B. capillosus</i> ATCC 29799	None	–	None	–	None	–
<i>B. cellulosolvens</i> ATCC 35603	None	–	None	–	None	–
<i>P. goldsteinii</i> DSM 19448	None	–	None	–	None	–

Complete and whole draft genome sequences accessible at <http://www.ncbi.nlm.nih.gov/>

B. Bacteroides, *P. Parabacteroides*

* *B. vulgatus* and *B. dorei* contain two short peptides with only a partial ferritin primary structure homologous, respective, to the N-terminus and C-terminus domains of FtnA3 (see text for details)