

ROLE OF THE IRON STORAGE FERRITINS DPS AND DPSL DURING THE
PROLONGED OXIDATIVE STRESS RESPONSE OF *BACTEROIDES FRAGILIS*

By

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Abstract

Bacteroides fragilis is a Gram negative anaerobe and member of the human intestinal tract microbiome. *B. fragilis* serves many beneficial roles within the intestinal tract; however, its translocation to the peritoneal cavity and the blood stream can result in peritonitis, intra-abdominal abscess formation, bacteremia and sepsis. We have shown that *B. fragilis* mediates both acute and Prolonged Oxidative Stress (POST) responses both *in vitro* and *in vivo*. This report characterizes the role and the genetic regulation of the iron storage proteins Dps and DpsL during the POST response. To test sensitivity to oxidative stress during the POST response a disk diffusion assay was developed using tert-butyl hydroperoxide (tBOOH). When the assay plates received aerobic exposure for three hours there was no zone of growth inhibition, whereas those kept under anaerobic conditions were highly sensitive to tBOOH. These results demonstrated an oxygen induced resistance to tBOOH that was mediated by prolonged aerobic exposure. To determine a mechanism for this POST induced resistance to tBOOH, a series of oxidative stress mutants were assayed. Only the Δdps mutant was sensitive to tBOOH after aerobic exposure indicating that Dps mediated the POST phenotype. Because of the similarities to Dps, the recently characterized DpsL (*bfr*) was

tested for a role in the POST response. The Δbfr mutant demonstrated resistance to tBOOH after aerobic exposure similar to wildtype (WT); however, when a double $\Delta dps \Delta bfr$ mutant was generated it demonstrated sensitivity to tBOOH that was greater than the Δdps mutant indicating that both Dps and DpsL play a role in the resistance phenotype. To explore the role that Dps and DpsL play in the survival of *B. fragilis* during infection, animal experiments were performed in the rat abscess model. Interestingly only the double $\Delta dps \Delta bfr$ mutant was attenuated in this model whereas neither of the single mutants showed a defect in competition experiments with WT. This indicated that both Dps and DpsL play a role in survival during infection.

To investigate genetic regulation during the POST response, it was essential to identify the second regulator of *dps* expression. It was previously shown that OxyR is a strong inducer of *dps* expression during acute oxidative stress; however, the $\Delta oxyR$ mutant was resistant to tBOOH after prolonged aerobic exposure similar to WT. This indicated that there was a second regulator of *dps* expression during the POST response. A known POST regulator, SigOF was investigated to determine if it played a role in this response. Similar to $\Delta oxyR$ the $\Delta sigOF$ mutant was resistant to tBOOH. Interestingly though a double $\Delta sigOF \Delta oxyR$ mutant was sensitive to tBOOH in the POST assay and *dps* expression was reduced as shown by qRT-PCR. These results strongly suggest that SigOF is the regulator responsible for *dps* expression during the POST response.

ROLE OF THE IRON STORAGE FERRITINS DPS AND DPSL DURING THE
PROLONGED OXIDATIVE STRESS RESPONSE OF *BACTEROIDES FRAGILIS*

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ROLE OF THE IRON STORAGE FERRITINS DPS AND DPSL DURING THE
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LIST OF ABBREVIATIONS

Ahp: alkyl hydroperoxide reductase
Bfr: bacterioferritin (unless referring to the *B. fragilis* *bfr* gene)
bfr: gene that encodes the DpsL protein of *B. fragilis*
BHIS: brain heart infusion supplemented with cysteine
CCF: commensal colonization factor
Cef: cefoxitin
Don: polysaccharide utilization locus mediates catabolism of N-glycans
Dps: DNA binding protein under starved state
DpsL: Dps like protein
ECF: extra cytoplasmic function
EcfO: ECF sigma factor
Erm: erythromycin
Fe²⁺: ferrous iron
Fe³⁺: ferric iron
Fis: nucleoid associated protein
Ftn: Ferritin
Fur: ferric uptake regulator
Gad: glutamate decarboxylase
Gls: glutaminase
H₂O₂: hydrogen peroxide
H-NS: nucleoid associated protein
Kat: catalase
Nig: nigrescin
O₂⁻: superoxide
OH[•]: hydroxyl radical
Osu: *B. fragilis* starch utilization operon
OxyR: oxidative stress regulator
PerR: peroxide resistance regulator
POST: Prolonged Oxidative Stress
PSA-H: Polysaccharide
Rbr: rubrerythrins
Reo: anti-sigma factor partner to EcfO
Rif: rifampicin
ROS: reactive oxygen species
SigOF: ECF sigma factor induced by oxygen
Sod: superoxide dismutase
tBOOH: tert-butyl hydroperoxide
Tet: tetracycline
Tps: thiol peroxidase
Tpx: thioredoxin peroxidase
Trx: thioredoxin
σ⁷⁰: *E. coli* Housekeeping sigma factor
σ⁵: *E. coli* stationary phase sigma factor

CHAPTER ONE: INTRODUCTION

The focus of this body of work was to determine the role that the DNA binding protein under starved state (Dps) plays during the Prolonged Oxidative Stress (POST) response in *Bacteroides fragilis*. Additional work showed that the Dps-like protein (DpsL) also plays a role during the POST response and that the differential regulation of these two genes contributes to increased survival during oxidative stress. The experimental data demonstrated a link between intracellular ferrous iron, oxidative stress, and the roles that Dps and DpsL play in converting and storing reactive iron leading to the protection of the cell during periods of oxidative stress. Overall these studies provide insight into the robust oxidative stress response and start to tease apart the differences between the acute and POST oxidative stress responses. This dissertation is organized in four chapters. Chapter one is a general overview of *B. fragilis* physiology, its role in intra-abdominal abscesses, the acute and POST oxidative stress responses, intracellular iron management, and the development of an extended exposure to air assay for the POST response. The second chapter then addresses specifically the roles of Dps and DpsL in providing protection both *in vitro* and *in vivo* during the POST response. Chapter three describes the identification of a second regulator that controls *dps* during the POST response. The last chapter is a summary of the work presented in this dissertation and future experimental directions for this project.

***B. fragilis* as a member of the normal intestinal flora**

The *Bacteroides* are Gram negative, non-spore forming, non-motile, anaerobic bacteria and members of the normal flora of the human intestinal tract. As a member of

the normal intestinal flora, *B. fragilis* plays many beneficial roles such as polysaccharide degradation, protection of the gut epithelia from colonization by pathogenic bacteria, development of the intestinal tract, maturation of mucosal and systemic immune systems, and transformation of toxic and mutagenic compounds (1-4). Recent work has focused on the role that the *B. fragilis* polysaccharide capsule plays in generation of an anti-inflammatory response believed to promote tolerance of this organism within the intestinal tract. The *B. fragilis* polysaccharide capsule A (PSA) has been shown to promote an anti-inflammatory response through stimulating the production of IL-10, an anti-inflammatory cytokine (5). This response is unique in that PSA is presented on a major histocompatibility complex class II (MHCII) and recognized by a subset of T-regulatory T-cells that are CD25, CD4, and Foxp3 positive driving an anti-inflammatory response (4, 6). Classic MHCII presentation occurs by presenting a peptide and it was later determined that the zwitterionic nature of PSA allows it to be recognized, bound, and presented in this manner (6, 7). This response promotes the tolerance of *B. fragilis* within the intestinal tract and is one of the many different tolerance mechanisms that have been discovered (7).

The human colon provides a nutrient rich and anaerobic environment that is favorable for the growth of *Bacteroides*. Interestingly though, recent work has demonstrated that the human colon is not uniformly anaerobic (8). An oxygen gradient is present from the submucosa to the lumen of the colon with the colonic crypts reaching up to 8% oxygen. This gradient can extend even further during nutrient absorption resulting in an expansion of the microaerophilic zone of the colon into the colonic lumen zone containing facultative anaerobic microbes (8). *B. fragilis* has been

known to colonize both the mucosal layer of the colon and inside the colonic crypts. The commensal colonization factor (CCF) has been shown to mediate colonization of the colonic crypts (9). This ability also was shown to promote survival during reestablishment of the *B. fragilis* population following challenge with antibiotics or the inflammatory response to invading pathogens. Interestingly colonization of the colonic crypts puts *B. fragilis* in an environment where oxygen levels can rapidly change thus exposing this organism to periods of oxidative stress. This may in part explain why *B. fragilis* has such a robust oxidative stress response and is one of the most aerotolerant anaerobes known (1).

Intra-abdominal abscess

The *Bacteroides* play many beneficial roles in the intestinal tract; however, in the event of an intestinal tear or a breakdown of the intestinal lining, translocation of the flora to the peritoneum occurs and disease can result. The *B. fragilis* group (*B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. uniformis*, and *Parabacteroides distosonis*) are the most frequently isolated anaerobes from cases of bacteremia, sepsis, meningitis, peritonitis, intra-abdominal abscesses, and other anaerobic infections, with *B. fragilis* being the predominantly isolated species (10). In the year 2000 these infections accounted for more than \$500 million in medical cost annually making them a significant burden on the health care system (11).

B. fragilis is best known for its persistence in intra-abdominal abscesses. When a perforation or compromise in the integrity of the intestinal lining occurs due to trauma, abdominal surgery or diseases such as appendicitis, perforated ulcer, diverticulitis, and

colon cancer, *B. fragilis* is able to translocate into the peritoneum. Translocation of the colonic natural flora leads to high levels of bacteria being present within the once sterile peritoneum. The majority of these organisms will be removed by the diaphragmatic lymphatics, the resident peritoneal macrophage population, and the influx of polymorphonuclear (PMN) cells. Those organisms that are not cleared are then subjected to encasement in fibrin matrices and the establishment of an abscess begins. As deposition of fibrinogen and the immune response continues formation of a mature abscess occurs. A mature abscess consists of a core which contains necrotic debris and surviving bacteria surrounded by a ring of neutrophils and macrophages, and a peripheral ring of fibroblasts and smooth muscles cells within a collagen capsule (11). At this point the immune system has effectively isolated the invading organisms however many organisms are unable to be effectively cleared from the abscess. These organisms can replicate within the abscess and reach a high cellular density. In the event the abscess ruptures high levels of bacteria can be released leading to bacteremia, sepsis, and in certain instances death (11-13)

B. fragilis is the most common anaerobic organism isolated from intra-abdominal abscesses and has been shown to possess many factors that promote its survival within the abscess. The most studied factor is the polysaccharide capsule. There are several reasons that the capsule is required for effective survival within the abscess. First is that the capsule allows for adherence to the peritoneal mesothelium which will prevent physical clearance of the organism after translocation into the peritoneum (14). The second reason is that the *B. fragilis* capsule is antigenically heterogeneous allowing for the production of eight distinctive polysaccharide capsule components (PSA-H) (15). A

plethora of work has been done on the roles that PSA plays during abscess formation (16-18). PSA is needed for intra-abdominal abscess formation by *B. fragilis* and a PSA knockout mutant is significantly attenuated in a murine abscess formation model (19). T cells are required for abscess formation and it has been shown that PSA and other bacterial components stimulate T cell dependent production of IL-17 a pro-inflammatory cytokine (16, 18, 20). IL-17 neutralization through use of an antibody prevents abscess formation *in vivo* demonstrating the importance of this response in abscess formation (21). Peritoneal macrophages produce IL-10 an anti-inflammatory cytokine in response to presence of *B. fragilis* in the peritoneum (21). This production of IL-10 has been shown to significantly decrease the severity of disease. IL-10^{-/-} mice experienced significantly higher levels of mortality in a murine model of intra-abdominal abscess formation showing the importance of production of IL-10 during abscess formation (21). This work has demonstrated the interesting and important role that *B. fragilis* plays during abscess formation and the significant impact this organism has on the progression of intra-abdominal abscesses.

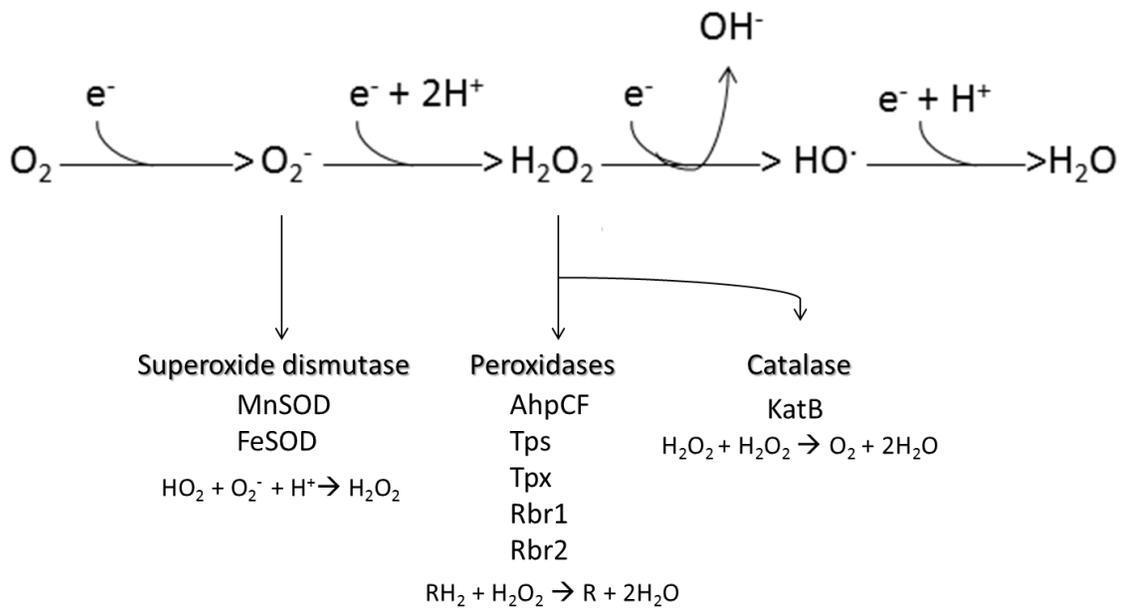
After establishment of an intra-abdominal abscess, *B. fragilis* is able to survive and reach high concentrations within the abscess. The abscess is a harsh environment where all nutrients must be derived from host factors. *B. fragilis* also has to survive the oxygenated peritoneum (6% O₂) and the immune system which are significant sources of oxidative stress (11, 22-24). Several factors promote survival during the establishment and within the abscess including proteases, neuraminidase, iron acquisition, hemolysins, and resistance to oxidative stress (1, 2, 25, 26). Recent work has shown that a polysaccharide utilization locus (Don) which mediates catabolism of

N-glycans on transferrin as the sole carbon source is important for survival within the abscess (27). The oxidative stress response also promotes survival of *B. fragilis* during formation of the abscess and provides a significant advantage for survival within the abscess. This was demonstrated by attenuation of the $\Delta oxyR$ and Δdps mutants in a murine abscess model (28). This dissertation will focus on the *B. fragilis* oxidative stress response and the role it plays in promoting survival within the abscess.

***B. fragilis* oxidative stress response**

B. fragilis has a robust oxidative stress response that results in the expression of detoxification enzymes, metabolic modifications, and a significant change in cell physiology (1, 28). This robust response prevents the accumulation of reactive oxygen species during periods of oxygen exposure and prevents cellular damage. *B. fragilis* is an anaerobic organism unable to grow in the presence of greater than 2% oxygen, however it is incredibly aerotolerant being able to survive for greater than 3 days in a fully aerobic environment (22% O₂) (29-31). Oxygen within the cytoplasm can quickly be converted to superoxide, hydrogen peroxide, and the damage inducing hydroxyl radicals Fig. 1.1.

Fig. 1.1 Hydroxyl radical formation in the cytoplasm. The conversion of oxygen to reactive oxygen species is shown from left to right. Molecular mechanisms for detoxification of the different reactive oxygen species are listed along with the *B. fragilis* proteins that utilize that mechanism.



The presence of oxygen within the cytoplasm in aerobic organism is beneficial as it can be used as a terminal electron acceptor in the generation of ATP. However, though energetically unfavorable, O_2 can accept electrons from donors within the cells leading to the formation of superoxide (O_2^-) Fig. 1.1. If superoxide receives an additional electron, hydrogen peroxide is produced (H_2O_2), and by accepting another electron a hydroxyl radical ($OH\cdot$) is produced. Conditions that favor the production of these reactive oxygen species (ROS) occur during substrate limitations during metabolism where oxygen is able to accept available electrons that would normally be funneled to the missing substrate (32). Glycyl-radical enzymes such as the anaerobic ribonucleotide reductase, pyruvate:formate lyase, and 2-ketobutyrate:formate lyase, are particularly susceptible to this when oxygen is present thus promoting the production of ROS (32, 33).

The production of ROS results in damage to the cells. Superoxide can react with iron-sulfur clusters producing H_2O_2 and an oxidized iron-sulfur cluster which is then hydrolyzed to free ferrous iron (Fe^{2+}) and results in an inactive iron sulfur cluster (32, 34). This inactivates the enzyme until the iron-sulfur cluster can be repaired. Hydrogen peroxide itself is unable to damage biomolecules due to the high energy of activation needed to react with these molecules. However, H_2O_2 readily will react with free ferrous iron to produce hydroxyl radicals through the Fenton Reaction. Hydroxyl radicals are very reactive and will react with most organic molecules causing damage close to the site of radical formation (32, 35). This makes the production of these ROS particularly dangerous to the cell and must be prevented or managed effectively for a cell to remain viable.

In order to prevent damage from ROS, *B. fragilis* has an extensive system of enzymes to detoxify the various ROS. As shown in Fig. 1.1, *B. fragilis* utilizes a superoxide dismutase (SOD) enzyme to convert superoxide to hydrogen peroxide. This enzyme is capable of utilizing either iron (Fe) or manganese (Mn) to catalyze this reaction (36). Many bacterial SODs cannot effectively substitute the metal but rather have two SODs, one that incorporates Fe and the second that utilizes Mn (32, 36). Additionally a series of peroxidases have been identified in *B. fragilis* that are responsible for keeping levels of H₂O₂ low. Alkyl hydroperoxide reductase (AhpCF) functions to convert hydrogen peroxide into water thus preventing the accumulation of H₂O₂. An *ahpCF* deficient strain of *B. fragilis* demonstrated sensitivity to peroxides as well as increased mutagenesis indicating that the role of this protein is to prevent peroxide driven damage to the cell (37). Further work has been done to show that the thioredoxin peroxidase (Tpx) and the thiol peroxidase (Tps) play a role in the oxidative stress response and in resistance to peroxides (38-40). Additionally there are two rubrerythrins encoded in the *B. fragilis* genome that may serve as peroxidases. In the closely related organism *Bacteroides thetaiotaomicron*, a similar number of peroxidases have been identified and it has been shown that the activity of these enzymes depends on the level of H₂O₂ within the cell. Under anaerobic conditions the rubrerythrins are the active peroxide scavengers until aeration occurs and the rubrerythrins become inactive and AhpCF and catalase (Kat) become the predominant peroxide scavengers (41). Catalase also was shown to play an important role in the oxidative stress response of *B. fragilis* where it promotes protection during high levels of peroxide exposure (31, 42). *B.*

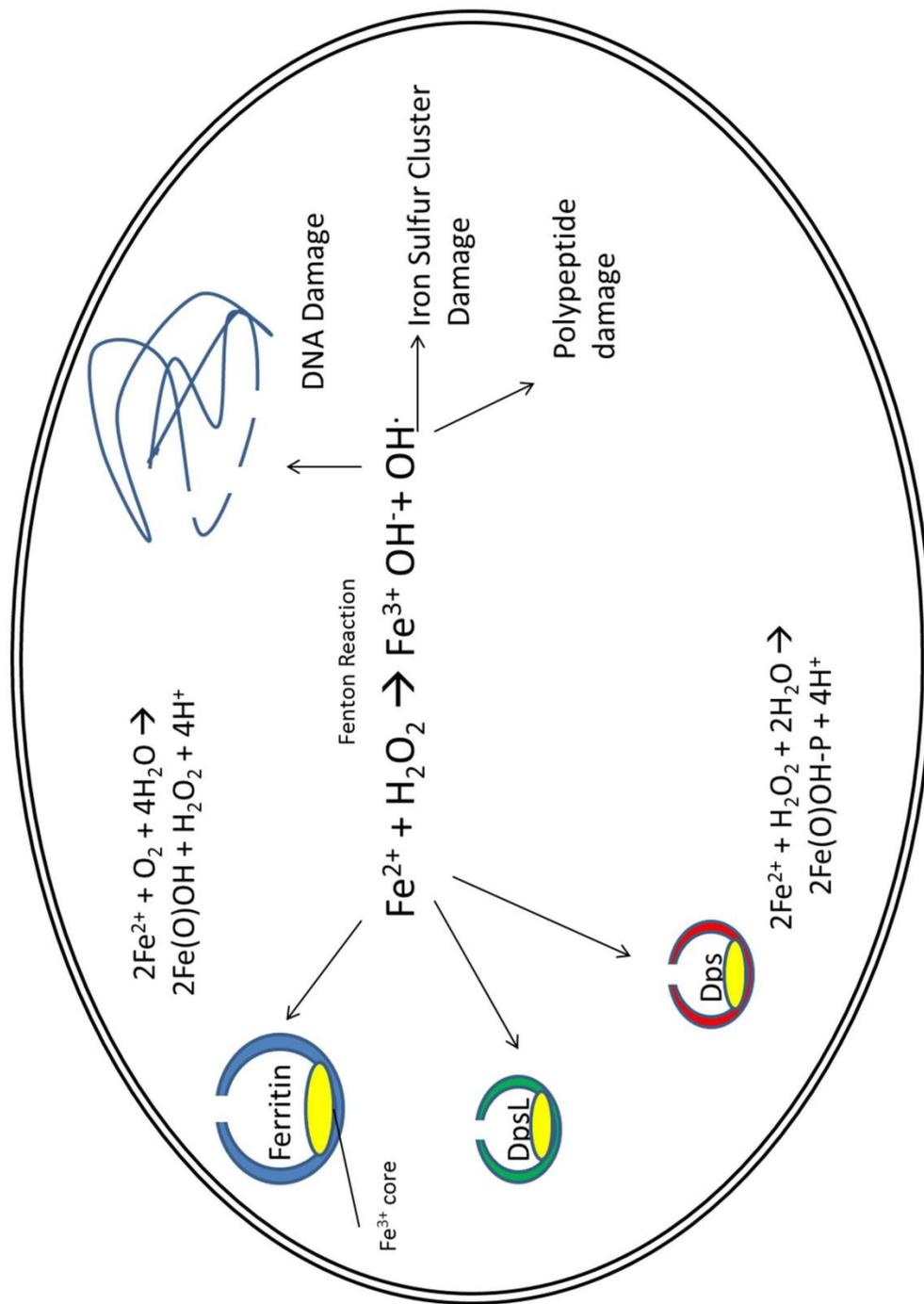
fragilis has a broad network of enzymes responsible for the degradation of ROS but there are additional systems that also contribute to survival during oxidative stress.

B. *fragilis* Iron storage proteins

As previously mentioned, the most damaging reaction that occurs when high levels of H_2O_2 are reached in the cytoplasm is the Fenton reaction and the generation of hydroxyl radicals (Fig. 1.2). Hydroxyl radicals ($\text{OH}\cdot$) can cause DNA damage, DNA strand cleavage, destruction of iron sulfur clusters, damage proteins, and possibly cause lipid peroxidation (32). The DNA damage is particularly dangerous as it is lethal to the cell. Unincorporated iron has been shown to bind to DNA thus when levels of hydrogen peroxide rise formation of the hydroxyl radical occurs near the DNA (43). This in turn puts the hydroxyl radical in close proximity to the DNA allowing for the abstraction of an electron from the ribose moiety resulting in a ribosyl radical that can react with oxygen leading to strand cleavage (32, 44). There are no known detoxification systems to resolve the hydroxyl radical in a safe manner within the cell. Therefore, it is necessary to prevent the generation of hydroxyl radicals by limiting the pools of Fe^{2+} and H_2O_2 .

As previously discussed there are many different mechanisms for the reduction of H_2O_2 in the cell. On the other hand, to prevent the accumulation of Fe^{2+} , cells utilize proteins belonging to Ferritin superfamily. These proteins effectively convert free soluble Fe^{2+} to the insoluble Fe^{3+} and store the Fe^{3+} within their core.

Fig. 1.2 The *B. fragilis* Iron Storage proteins. The Fenton reaction is shown which promotes the production of hydroxyl radicals. The hydroxyl radical is capable of damaging macromolecules in the cell. As shown, *B. fragilis* proteins belonging to the ferritin-like superfamily are able to remove reactive Fe^{2+} from the cytoplasm preventing the generation of hydroxyl radicals.



This process effectively removes the Fe^{2+} from the cytoplasm until the cytoplasm is returned to a reduced state. Bacteria possess four different types of ferritin proteins Ferritins, Bacterioferritins, Dps-like, and Dps. All of these proteins have very similar structure in that their subunits consist of a bundle of four alpha helices that form two homologous pairs of anti-parallel helices (45). The subunits then assemble to form a large spherical protein with a hollow center. These four types function in a similar manner to convert Fe^{2+} to Fe^{3+} but they accomplish this in different ways.

Ferritins and Bacterioferritins utilize O_2 to catalyze the conversion of Fe^{2+} into Fe^{3+} and store it in a ferri-oxyhydroxide mineral within their core Fig. 1.2 (45, 46). This conversion is achieved initially within the ferroxidase center which is located within the channel formed by the four alpha helices. These proteins consist of 24 subunits that form the largest hollow sphere of the Ferritin family (47). This allows them to store large amounts of iron (3000-4000 atoms) within the core (45). Bacterioferritins are very similar to ferritins but they incorporate up to 12 heme moieties within their spherical shell (48). The role of this heme group is unknown but it is believed to allow for release of iron from the core through a reduction mechanism (49). In aerobic bacteria ferritins and bacterioferritin are believed to store iron when excess levels become present in the cytoplasm thus limiting the amount of iron available to produce hydroxyl radicals when oxidative stress occurs in the cells.

Dps proteins display major differences from the ferritins and bacterioferritins. Fully functional Dps consists of a cluster of 12 subunits which form a spherical structure with a hollow center. This smaller structure only allows for the storage of up to 500 iron

atoms within the core (45). Unlike the ferritins and bacterioferritins, the ferroxidase site is formed within the interface of two subunits as opposed to being contained within the four helix bundle as seen in ferritins and bacterioferritins. This ferroxidase site also catalyzes a different reaction as demonstrated in Fig.1.2 hydrogen peroxide is used to convert ferrous iron to ferric iron. The coordination between the two iron binding residues within the ferroxidase center promotes a two step process that results in conversion of 2 Fe²⁺ to 2 ferrihydrite-like molecules by consuming one molecule of hydrogen peroxide while avoiding the production of a hydroxyl radical (50).

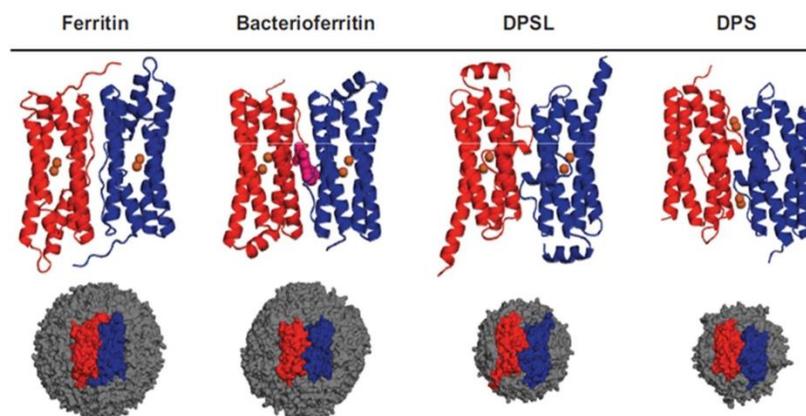
Dps is well known for its ability to protect DNA from damage during oxidative stress and this protective mechanism has been demonstrated in many different organisms. The association between Dps and DNA is well understood in the model organism *Escherichia coli*. The *E. coli* Dps binds to DNA non-specifically by a mechanism that is mediated through conserved residues of the N-terminus (51). This assists in localizing Dps to the DNA where under oxidative stress any free iron associated with the DNA can be converted to the non-reactive ferric form (51, 52). It is important to note that while not all Dps proteins have the ability to bind DNA they are still able to protect the DNA from oxidative stress damage (53-55). The mechanism of DNA binding is not well conserved and there are several unique mechanisms for DNA binding or association. On the other hand the ferroxidase activity and the resistance to oxidative stress is well conserved pointing to the importance of the ferroxidase activity for protection of the cells (56, 57).

The recently identified Dps-like (DpsL) class of proteins demonstrate similarities to both the Dps and Bacterioferritin classes. As shown in Fig. 1.3, the DpsL proteins have a similar tertiary protein structure to the ferritin and bacterioferritin with conserved α -helices and the ferroxidase center buried within the channel formed by the cluster of helices. The DpsL protein also contains a dimetal binding site similar to ferritin and Bacterioferritin (58). However, the DpsL protein assembles into a 12 subunit hollow sphere and has a preference for H_2O_2 in the ferroxidase reaction which is similar to Dps (58). This protein was first identified in the archaea *Sulfolobus solfataricus* and *Pryococcus furiosus* with roles involved in protecting the cells from oxidative stress damage (59, 60). Given the unique characteristics of this protein and the work done on the *S. solfataricus* DpsL protein, it has been hypothesized that this protein is capable of a peroxidase activity where Fe^{2+} is used to catalyze a reaction to detoxify H_2O_2 while simultaneously reducing the free iron within the cell, a function similar to Dps (58).

B. fragilis contains three homologues belonging to the Ferritin superfamily of proteins. They belong to the ferritin, DpsL, and Dps classes of proteins and several studies have characterized the role these proteins play in the oxidative stress response. Many prominent members of *Bacteroides* contain several homologues of ferritin family proteins but the specific roles that these proteins play in survival of these organisms as well as the specific reason why this genus requires so many is unknown (61).

Fig. 1.3 Characteristics of the ferritin family proteins. DPSL proteins combine features from other members of the ferritin superfamily. All ferritins assemble into hollow, spherically shaped oligomers made up of either 24 subunits (ferritins and bacterioferritins) or 12 subunits (DPSLs and DPS proteins). A representative member of each class of the ferritin superfamily from the bacterial domain of life is shown as both the complete oligomer, and as a two subunit assembly: *Helicobacter pylori* ferritin (PDBID 3EGM), *E. coli* bacterioferritin (1BCF) *Bacteroides fragilis* DPSL with its N-terminal and extended D helices (2VZB) and bacterioferritin (1BCF), *Bacteroides fragilis* DPSL with its N-terminal and extended D helices (2VZB) and *Bacillus brevis* DPS (1N1Q). DPSLs share the dodecameric (12-mer) quaternary structure of DPS proteins. However, the DPSL ferroxidase site (red spheres) is buried within the subunit, similar to ferritins and bacterioferritins, rather than at the subunit interface like DPS. Furthermore, residues that constitute the DPSL ferroxidase site most closely resemble those in bacterioferritin, with the addition of a conserved cysteine pair. Note that bacterioferritins differ from ferritin, DPS and DPSL by incorporating a heme at the subunit interface (pink) (66). *Human ferritin has been shown to bind DNA in the presence of iron (116). †Not all Dps proteins are able to bind DNA (see text for further explanation).

This figure is originally published in the Journal of Bacteriology. Gauss GH, Reott MA, Rocha ER, Young MJ, Douglas T, Smith CJ, Lawrence CM. Characterization of the *Bacteroides fragilis* bfr gene product identifies a bacterial DPS-like protein and suggests evolutionary links in the ferritin superfamily. J Bacteriol. 2012 Jan;194(1):15-27. doi: 10.1128/JB.05260-11. Copyright © 2012, American Society for Microbiology **Appendix A**



| | Ferritin | Bacterioferritin | DpsL | Dps |
|--|--------------|------------------|-------------------------------|-------------------------------|
| Number of subunits | 24 | 24 | 12 | 12 |
| Ferroxidase core | Similar | Similar | Similar | Unique |
| Predominant Role | Iron Storage | Iron storage | Unknown | DNA protection |
| Preferred ferroxidase substrate | Oxygen | Oxygen | H ₂ O ₂ | H ₂ O ₂ |
| Iron capacity (in atoms) | 4500 | 4500 | Unknown | 500 |
| DNA Binding | Yes/No* | No | No | Yes/No† |

The *Bacteroides* have more ferritin homologues than any other known bacterial species (61). In the case of *B. thetaiotaomicron* homologues for three ferritins, one DpsL, and one Dps are present within its genome. Many of the other *Bacteroides* species share a similar number of ferritin family homologues (further discussed in Chapter 2).

The role that the three Ferritin family homologues FtnA, Dps, and DpsL play in the cell has been investigated in *B. fragilis*. The majority of the work on these proteins has been to characterize their genetic regulation (to be discussed in the next section). Ferritins play central roles in the oxidative stress response, management and storage of excess iron, and are expressed during stationary phase. Organisms that have multiple ferritins similar to *B. fragilis* control their expression under specific conditions. In the case of *Salmonella enterica* sv. Typhimurium there are four proteins belonging to the Ferritin family FtnA, FtnB, Bacterioferritin (Bfr), and Dps. Bfr serves as the predominant ferritin for managing elevated levels of Fe^{2+} within the cell and is the main ferritin involved in iron storage. FtnA also appears to be specific for the management of high levels of Fe^{2+} within the cells while Dps and FtnB are involved in the oxidative stress response (62). The regulation of and the specific characteristics of these ferritins allows for *S. enterica* to utilize them under specific conditions. This specialization may also be the reason why *B. fragilis* and the *Bacteroides* have several ferritins.

Characterization of the *B. fragilis* Ferritin homologue FtnA showed that expression of the *ftnA* gene product occurred only in the presence of oxygen and not under excess iron conditions under anaerobic conditions (63). This makes sense as ferritins utilize oxygen to catalyze the conversion of Fe^{2+} to Fe^{3+} and without it the protein would be non-functional. Characterization of a $\Delta ftnA$ mutant however showed

that oxygen exposure had no effect on the viability of this strain. It was not until a multiple $\Delta ftnA \Delta dps \Delta oxyR$ mutant was generated that a loss in cell viability in response to oxygen was seen (63). This has been seen in other organisms where defects in resistance to oxidative stress and loss of iron storage capability are not seen until inactivation of multiple ferritin family genes (62, 64, 65). Overall this work indicated that FtnA functions to prevent the accumulation of Fe^{2+} during exposure to oxygen and thus demonstrated a role in the oxidative stress response.

The *B. fragilis* DpsL was originally annotated as a bacterioferritin because of the conserved iron binding site motif and it was assigned the gene name *bfr*. However, later work demonstrated that the *B. fragilis bfr* gene actually encodes a DpsL (66). For clarification purposes in this document when the gene name *bfr* is used it refers to the gene that encodes the *B. fragilis* DpsL. Studies with DpsL showed a role in the oxidative stress response and that the purified protein is able to protect DNA from cleavage in the presence of iron and hydrogen peroxide, an activity that is similar to Dps proteins of other organisms (51, 66). It was also shown that this protein formed a 12 subunit complex with preferential use of H_2O_2 as a substrate for ferroxidase activity. Characterization of the Δbfr mutant further showed a deficiency in the ability to resist oxidative stress inducing agents as well as to survive prolonged exposures to oxygen (66). This work demonstrated the importance of DpsL in the oxidative stress response and was the first ever DpsL to be identified and characterized in bacteria.

To date most of the work with the *B. fragilis* Dps has focused on the regulation of the *dps* gene by the oxidative stress regulator OxyR. Interestingly it has been shown that the *B. fragilis* Dps does play a role in abscess formation as demonstrated by the

attenuation of the Δdps mutant in a mouse abscess model indicating a role in survival of this organism during infection (28). The work described in this dissertation has further characterized the role of Dps in survival of *B. fragilis* in both the acute and POST oxidative stress responses (see Chapter 2).

Overall the role of the ferritin family proteins in *B. fragilis* is very important to the cells ability to survive. This survival is directly influenced by the tight regulation and control of these various ferritins by a wide variety of regulators that coordinate their expression during the oxidative stress response. It is this regulation that allows *B. fragilis* to resist oxidative stress and be one of the most aerotolerant anaerobic organisms (1).

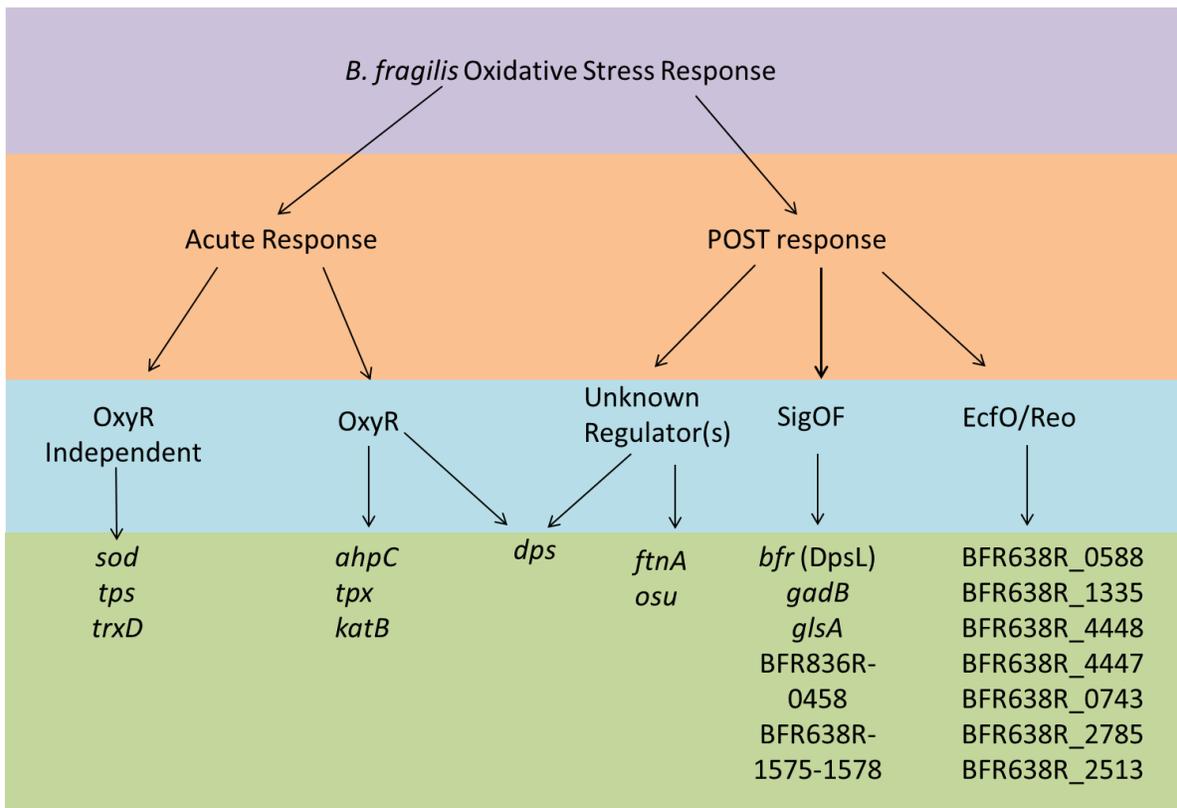
Regulation of the *B. fragilis* oxidative stress response

Regulation of the oxidative stress response in *B. fragilis* is complex and involves many different regulators some of which have been identified and others which have not. When *B. fragilis* is first exposed to oxygen a rapid induction of 28 peptides occurs as part of the acute oxidative stress response (28). This response occurs within the first five minutes of exposure and the predominant function of this response appears to be to prevent the accumulation of ROS. If oxidative stress is prolonged then the cells undergo a significant change in cell physiology and metabolism which is driven by a genome wide change in transcription (45% of the genome) patterns known as the POST response (28). This response is designed to put the cell in a state where it is able to resist oxidative stress for extended periods of time by inducing carbohydrate utilization systems, altering central metabolism, transport and efflux and repressing DNA

synthesis, translation, and membrane biogenesis (28). The coordination and the genetic regulation of these two responses are of key interest to understanding the *B. fragilis* oxidative stress response and how it promotes survival of this organism during infection.

Regulation of the acute OSR response has focused on the role of OxyR in preventing the accumulation of H₂O₂. As shown in Fig. 1.4 a major branch of the acute response involves the activation of OxyR and the rapid induction of genes that focus on the detoxification of H₂O₂ (28, 67, 68). The activation of OxyR requires the oxidation of two conserved cysteine residues that form a disulfide bridge which then promotes transcription of the OxyR regulon (32). In *B. fragilis* the activation of OxyR leads to the transcription of *aphCF*, *tpx*, *katB*, and *dps* all of which are directly responsible for detoxifying H₂O₂ within the cell (28, 68, 69). Also during the acute OSR the induction of *sod*, *tps*, *trxD*, and other genes occurs in an OxyR independent manner. Several of these genes are associated with the detoxification of ROS (*sod* and *tps*) whereas others are involved in repair and metabolism (28, 39, 69, 70). Together the OxyR dependent and OxyR independent branches of the acute response provide the cell with a quick and efficient response to rapid rises in oxidative stress protecting the cell from any immediate damage.

Fig. 1.4 *B. fragilis* oxidative stress response. A flow chart demonstrating the regulation of the oxidative stress response. The *B. fragilis* OSR is separated into the acute and post response. The blue represents known and unknown regulators involved in the control and expression of the genes listed in the green background. Lines indicate which genes are controlled by the regulator and what response that regulator is involved in. Overlap between the two responses can be seen in the regulation of the *dps* gene (28, 38, 39, 63, 68, 69, 71).



With a greater than 45% change in transcription patterns, the regulation of the POST response is quite complex. A major focus of research has been on the regulation of this response by extra cytoplasmic function (ECF) sigma factors that are induced during exposure to oxygen (28). These ECF sigma factors were designated SigOA-K (SigOD was renamed EcfO) and investigation of EcfO and SigOF has been pursued. EcfO and its anti-sigma factor partner Reo have been shown to play roles in resistance to oxidative stress inducing agents as well as survival during prolonged exposure to oxygen (71). An increase in induction of *ecfO* is seen starting after one hour oxygen exposure indicating this ECF sigma factor plays a role in the POST response. The EcfO regulon has been determined and contains several genes that share homology to a genetic locus for production of nigrescin (Nig), a bacteriocin secreted by *Prevotella nigrescens* (71, 72). As shown in Fig. 1.4 the regulon also includes a member of the Radical SAM family which is a large protein family containing iron sulfur clusters and having diverse functions involved in protein modifications and general metabolism. Additionally this regulon also contains secreted lipoproteins of unknown function. Although the function of the EcfO regulon is unknown it has been shown to play a role in resistance to oxidative stress and play a part in the POST response. Future work will hopefully shed light onto the specific activities of this regulon.

Another ECF sigma factor that is expressed during the post response is SigOF. Expression microarray analysis has determined the SigOF regulon includes *bfr* (DpsL), a glutamate decarboxylase *gadB*, glutaminase *glsA*, a putative transporter BF638R_0458, and an operon containing several putative fimbrin associated anchor proteins (unpublished data Ndamukong and Smith). Of particular interest is the

transcriptional induction of *bfr* (DpsL) after prolonged oxygen exposure. As will be demonstrated in Chapter 2, DpsL and Dps contribute to resistance to tert-butyl hydroperoxide by preventing the accumulation of free Fe²⁺ within the cell during prolonged exposure to oxygen. This could indicate that SigOF is controlling the expression of *bfr* (DpsL) during the POST response and may be playing a significant role in promoting long term survival of *B. fragilis* when exposed to oxygen. Further work is needed to determine the exact role that SigOF plays to promote survival of *B. fragilis* during the POST response.

Understanding the regulation and control of the POST response has resulted in a greater understanding of the physiological processes that the cells undertake in order to survive prolonged oxygen exposure. However, there still are several important genes induced during the POST response for which no regulation have been identified Fig. 1.4. Studies of *ftnA* and the oxygen induced starch utilization operon, *osuA*, have demonstrated important roles for both in survival during oxygen exposure (63, 73) however the identity of regulators that control the expression of these genes during the POST response remains elusive. Identification of these regulators will provide great insight into the many different processes that *B. fragilis* utilizes for survival during the POST response.

The focus of the work in this dissertation has been to determine the roles that Dps and DpsL play in the POST response. To achieve this, an assay was designed that demonstrated resistance to tert-butyl hydroperoxide (tBOOH) only after prolonged oxygen exposure. Using this assay Dps and DpsL were identified as contributors to the increased resistance to tBOOH. Tert-butyl hydroperoxide is not easily detoxified within

B. fragilis as indicated by extreme sensitivity to this agent (39). This sensitivity may be due to several factors, the first being that tBOOH cannot fit within the cleft of catalase thus preventing detoxification by this enzyme (74). Another property of tBOOH that makes it useful for this study is that activation of OxyR which requires oxidation of two cysteine residues (68), is less efficient due to inefficient oxidation of cysteine by tBOOH (75). This property of tBOOH also makes detoxification by AhpCF, Tpx, Tps, and most peroxidases that utilize a mechanism of oxidation of a cysteine residue inefficient in detoxifying tBOOH. A final factor makes tBOOH an attractive agent for this study is the high affinity for transition metals, such as Fe²⁺ and Cu²⁺, which it reacts with to form hydroxyl radicals and damage macromolecules (76). Additionally tBOOH has been shown to have a Fe²⁺ dependent mechanism of producing methyl radicals which has a mutagenic effect on DNA (77, 78). The tert-butyl hydroperoxide resistance phenotype has been very useful in demonstrating a role for Dps and DpsL in the POST response in an iron dependent manner and showing the importance of a second regulator of *dps* expression during the POST response.

CHAPTER TWO: DPS AND DPSL MEDIATE SURVIVAL *IN VITRO* AND *IN VIVO* DURING THE PROLONGED OXIDATIVE STRESS RESPONSE IN *BACTEROIDES FRAGILIS*

Abstract

Bacteroides fragilis is a Gram negative anaerobe and member of the human intestinal tract microbiome where it serves many beneficial roles. However, translocation of the organism to the peritoneal cavity can lead to peritonitis, intra-abdominal abscess formation, bacteremia, and sepsis. During translocation, *B. fragilis* is exposed to increased oxidative stress from the oxygenated tissues of the peritoneal cavity and the immune response. In order to survive *B. fragilis* mounts a robust oxidative stress response consisting of an acute and a Prolonged Oxidative Stress (POST) response. This report demonstrates that the ability to induce high levels of resistance to *tert*-butyl hydroperoxide (tBOOH) after extended exposure to air can be linked to the POST response. Disk diffusion assays comparing wild type to the Δdps and a double $\Delta dps \Delta bfr$ mutant showed greater sensitivity of the mutants to tBOOH after exposure to air suggesting that Dps and DpsL play a role in the resistance phenotype. Complementation studies with *dps* or *bfr* (encoding DpsL) restored tBOOH resistance suggesting a role for both of these ferritin-family proteins in the response. Additionally, cultures treated with the iron chelator dipyriddy were not killed by tBOOH indicating Dps and DpsL function by sequestering iron to prevent cellular damage. An *in vivo* animal

model showed that the double $\Delta dps \Delta bfr$ mutant was attenuated indicating that management of iron is important for survival within the abscess. Together these data demonstrate a role for Dps and DpsL in the POST response which mediates survival *in vitro* and *in vivo*.

Importance

B. fragilis is the anaerobe most frequently isolated from extraintestinal opportunistic infections but there is a paucity of information about the factors that allow this organism to survive outside of its normal intestinal environment. This report demonstrates that the iron storage proteins Dps and DpsL protect against oxidative stress and they contribute to survival both *in vitro* and *in vivo*. Additionally this work demonstrates an important role for the POST response in *B. fragilis* survival and provides insight into the complex regulation of this response.

Introduction

The *Bacteroides* are members of the normal intestinal microbiome of humans. The intestine is a consistent and favorable environment that provides continuous access to nutrient sources for these strict anaerobic organisms. The *Bacteroides* play many important roles in maintaining a healthy intestinal tract such as 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, and transformation of toxic and mutagenic compounds (1-4). However, when the integrity of the intestinal wall is breached due to trauma, abdominal surgery, or diseases such as appendicitis, perforated ulcer, diverticulitis, and colon cancer

translocation of the normal flora into the peritoneal cavity can result in peritonitis and establishment of an intra-abdominal abscess. The inability of the host immune system to resolve the abscess can lead to bacteremia, sepsis, and in certain instances death (12, 13). *B. fragilis* is the most common anaerobe isolated from intra-abdominal abscesses and it has been demonstrated to possess many factors that promote its survival outside of the intestinal tract such as capsular polysaccharides, proteases, neuraminidase, iron acquisition, hemolysins, and resistance to oxidative stress (1, 2, 25, 26). Oxidative stress occurs immediately when *B. fragilis* translocates from the anaerobic intestine to the more oxygenated (6% O₂) peritoneal cavity, and there is additional oxidative stress resulting from the immune response and PMN recruitment to the site of infection (11, 22-24). Thus the oxidative stress response is needed for survival during abscess formation (28).

The *B. fragilis* oxidative stress response is a well-coordinated global response (28). Numerous studies have identified genes and proteins involved in the acute oxidative stress response, many of which are controlled by the LysR family transcriptional regulator, OxyR (28, 31, 39, 68). This response occurs rapidly after exposure to H₂O₂ or oxygen and involves activation of OxyR followed by induction of its regulon whose gene products are aimed at peroxide detoxification such as catalase (*katB*), alkyl hydroperoxide reductase (*ahpCF*), the non-specific DNA binding protein Dps (*dps*) and others (28, 37, 68). If oxidative stress and exposure to air are extended for an hour or more, a global shift in transcription occurs referred to as the Prolonged Oxidative Stress (POST) response aimed at remodeling cell physiology. This shift alters transcription of nearly 45% of the genes within the genome with significant changes in

the expression of genes for carbohydrate utilization, central metabolism, transport, and transcriptional regulators (28). These changes allow *B. fragilis* to survive for extended periods in air, (>100 hours) but specific regulatory factors that control the response have not yet been identified.

Management of intracellular iron availability is a key component of the oxidative stress response. The ferritin family of proteins is responsible for removing excess ferrous iron (Fe^{2+}) from the cytoplasm of cells to prevent generation of the damage inducing hydroxyl radicals via the Fenton reaction (52). These proteins bind and convert Fe^{2+} to non-reactive insoluble ferric (Fe^{3+}) iron thus preventing production of hydroxyl radicals (51, 79, 80). Members of this family include ferritin, bacterioferritin, Dps, and the recently discovered Dps-like (DpsL) proteins (52, 58). Dps protects cells from oxidative stress damage and shows a strong induction in response to oxidative stress in many organisms (28, 56, 81, 82). The *B. fragilis dps* gene has been shown to be rapidly induced by the oxidative stress regulator OxyR in the acute oxidative stress response however an OxyR independent induction of *dps* transcription also has been reported (68). Those results demonstrated that activity of a *dps::xyIB* transcriptional fusion was significantly induced during aerobic incubation of the ΔoxyR mutant. This result was only seen for *dps* expression and not with other members of the OxyR regulon indicating a second regulator was responsible (68). The DpsL protein has been shown to have very similar structure and function to Dps in both the archaea *Sulfolobus solfataricus* and *B. fragilis* (58, 66). *B. fragilis* DpsL, the first identified in bacteria, is induced by oxygen and has been shown to play a protective role during periods of oxidative stress (28, 66). The DpsL gene was originally incorrectly annotated as a

bacterioferritin and was designated *bfr*, however later structural studies determined it actually encodes a DpsL protein (66). *B. fragilis* does not have a true bacterioferritin. In this report we used an assay to examine the protective response induced by extended exposure to air. The results demonstrated a role for Dps and DpsL in the POST response which promotes survival both *in vitro* and *in vivo*. The protective role that Dps and DpsL play during the POST response is linked to the presence of ferrous iron indicating that these proteins function to convert and store reactive ferrous iron to non-reactive ferric iron. Additionally this work indicates that transcriptional control of *dps* is mediated by a second unknown regulator during the POST response and these data are consistent with previous findings (28, 68).

Materials and Methods

Bacterial strains and growth conditions. *Bacteroides* strains used in this study are listed in Table 2.1. All strains were grown anaerobically in brain heart infusion broth supplemented with hemin, cysteine, and NaHCO₃ (BHIS) unless otherwise noted (83). Rifampicin (20 µg/ml), gentamicin (50 µg/ml), tetracycline (5 µg/ml), ceftiofur (25 µg/ml), and erythromycin (10 µg/ml) were added to the media when needed.

Table 2.1 Strain and plasmid used in the work presented in Chapter 2.

| Strains or plasmids | Phenotype and/or genotype ^a | Reference or source |
|---------------------|---|---------------------|
| <i>Bacteroides</i> | | |
| IB-101 | <i>B. fragilis</i> 638R clinical isolate, Rif ^r | (84) |
| IB-260 | IB-101 $\Delta kat::tetQ$, Rif ^r Tet ^r | (31) |
| IB 298 | IB-101 $\Delta oxyR::tetQ$, Rif ^r Tet ^r | (68) |
| IB 336 | IB-101 $\Delta dps::tetQ$, Rif ^r Tet ^r | (63) |
| IB-430 | IB-101 $\Delta aphc::tetQ$ Rif ^r Tet ^r | This Study |
| IB-445 | ADB77 (isogenic with IB101) reverted to <i>thyA</i> ⁺ , <i>Atps</i> Rif ^r | (38) |
| IB-542 | IB-336 $\Delta bfr::cfx$, Rif ^r Tet ^r Cfx ^r | This Study |
| IB-567 | IB-542 pFD288:: <i>bfr</i> , Rif ^r Tet ^r Cfx ^r Erm ^r <i>bfr</i> ⁺ | This Study |
| IB-572 | IB-336 pFD288:: <i>dps</i> , Rif ^r Tet ^r Erm ^r <i>dps</i> ⁺ | This Study |
| IB-573 | IB-542 pFD288:: <i>dps</i> , Rif, Tet ^r Cfx ^r Erm ^r <i>dps</i> ⁺ | This Study |
| BER-74 | IB-101 $\Delta bfr::cfx$ Rif ^r , Tet ^r | (66) |
| IB-102 | <i>Bacteroides uniformis</i> , VPI strain 006-1 (ATCC 8492) Rif ^r | (85) |
| IB-103 | <i>Bacteroides ovatus</i> , VPI 0038 (ATCC 8433) | (86) |
| IB 114 | <i>Bacteroides fragilis</i> , ATCC 25285, clinical isolate, Rif ^r | (28) |
| IB-116 | <i>Bacteroides thetaiotaomicron</i> , VPI 5482 (ATCC 29148) | (86) |
| IB-351 | <i>Bacteroides vulgatus</i> , ATCC 8482 | (86) |
| IB-568 | <i>Bacteroides caccae</i> VPI 3452A (ATCC 43185) | (87) |
| BER-37 | <i>Parabacteroides distasonis</i> , clinical isolate CLA 348 | (88) |
| BER-39 | <i>Parabacteroides merdae</i> , VPI T4-1 (ATCC 43184) | (87) |
| <i>E. coli</i> | | |
| DH10B | F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi 80lacZAM15$ $\Delta lacX74$ <i>recA1</i> <i>endA1</i> <i>araD139</i> $\Delta(ara leu)$ 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>nupG</i> λ - | Invitrogen |
| HB101::RK231 | HB-101 containing RK231, Km ^r Tc ^r St ^r | (89) |
| Plasmids | | |
| pFD288 | (Sp ^r), Erm ^r , oriT, pUC19::pBI143 8.8-kb shuttle vector | (90) |
| pFD516 | <i>Bacteroides</i> suicide vector derived by deletion of pBI143 from pFD288, (Sp ^r), Erm ^r | (90) |

Table 2.1: Strains and Plasmids used in this study

^aErm^r, erythromycin resistance; Cfx^r, cefoxitin resistance; Rif^r rifampicin resistance; Tet^r, tetracycline resistance; Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance. For *Bacteroides-E. coli* shuttle vectors, parentheses indicate antibiotic resistance expression in *E. coli*.

Construction of mutant strains. All primer sequences used for genetic manipulations are listed in the supplemental material Table 2S-1. Briefly, the $\Delta ahpC$ mutant was constructed by PCR amplification of the N-terminal fragment of *ahpC* using oligonucleotides containing EcoRI sites. The fragment was cloned into a suicide vector, pFD516. A C-terminal fragment was amplified using the same approach except oligonucleotides contained a BamHI recognition site at the 5' end and *SphI* at the 3' end. This was then cloned into pFD516 containing the N-terminal fragment. A 2.2kb tetracycline cassette (*tetQ*) was inserted in between the N and C-terminal fragments using the *SacI* site. This plasmid was then mobilized into *B. fragilis* IB-101 and exconjugants were selected on BHIS plates containing rifampicin, gentamicin, and tetracycline (91). Sensitivity to erythromycin was determined, and PCR was performed to confirm the double-crossover allelic exchange of *ahpC::tetQ* mutation in strain IB430.

Construction of the double $\Delta dps \Delta bfr$ mutant, IB-542, was performed by mobilizing the BER-74 (66) mutational construct into IB-336 (63). Mutants were selected on rifampicin, gentamicin, and cefoxitin. PCR was performed to confirm the double-cross over allelic exchange of *bfr::cfxA*. Complementation of the Δdps mutation in IB-336 and IB-542 was done as follows. The full length *dps* gene including its native promoter was excised with *SphI* and *EcoRI* from plasmid pFD750, cloned into pFD288, and then mobilized into IB-336 and IB-542 respectively (68). Complemented mutants were selected on erythromycin to create IB-572 and IB-573 respectively. Complementation of Δbfr (IB-542) was performed by PCR amplification of full length *bfr* gene including its promoter and insertion into pFD288. Complemented mutants were plated on erythromycin to confirm presence of the plasmid.

Disk Diffusion assays. Disk diffusion assays were performed as previously described (66). In brief, 100 μ l of overnight culture was spread on BHIS plates (without cysteine) and a 6-mm filter disk was placed in the center of the plate. The disks were then saturated with 10 μ l of 55 mM tBOOH. Plates either were immediately incubated anaerobically at 37°C or they received 3 hours of aerobic incubation at 37°C prior to anaerobic incubation. Following overnight anaerobic incubation, the diameters of the zones of growth inhibition were measured, and the results were reported as the average of at least two independent experiments performed in triplicate. A Student's two tailed *t*-test was performed to determine significant differences between populations when appropriate.

Cell viability assays. Cell viability assays were performed by growing cultures to an OD₅₅₀ of 0.3 in BHIS without cysteine. Cultures were then split and half of the culture was shaken at 250 rpm in air at 37°C for three hours. The remaining half was kept under anaerobic conditions and challenged with 500 μ M tBOOH. Samples were taken over time and washed three times with BHIS to remove tBOOH. These samples were then serially diluted and plated to determine number of CFU/ml. After three hours of aerobic shaking, the other half of the culture was challenged with 500 μ M tBOOH. Samples were taken and processed as described above. Results are reported as an average of two independent experiments performed in triplicate.

Cell viability assays performed using 2,2'-dipyridyl (Sigma-Aldrich, St. Louis) were done as follows. Cultures were grown in BHIS without cysteine to an OD₅₅₀ of 0.3 and then split. All cultures were kept under anaerobic conditions, but half of the culture was treated with 2,2'-dipyridyl (300 μ M) 30 minutes prior to challenge with 500 μ M

tBOOH. Samples were taken over time and CFU/ml was calculated as described above. Results are reported as an average of two independent experiments performed in triplicate.

In vivo competition assays. The rat tissue cage infection model has been described previously (26, 92). Briefly a perforated sterilized ping pong ball is surgically implanted into the peritoneal cavity of an adult male Sprague-Dawley rat and allowed to encapsulate for 4-5 weeks. During this time the ball becomes encapsulated in connective tissue, the tissue becomes vascularized, and the ball fills with sterile serous fluid (~25 mL per ball). Competition assays were performed in this model as previously described (27). In brief, overnight cultures were diluted in PBS [50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4] and mixed in a 1:1 ratio of wild type (IB-101) and mutant (Δdps , Δbfr , or $\Delta dps \Delta bfr$) to a total of 1×10^5 CFU/ml as a standard inoculum. Four ml of inoculum were injected into the tissue cage. Samples were taken at specific time points, serially diluted and plated on rifampicin and gentamicin. After 2-3 days incubation, 200 colonies from each sample were tested for growth on BHIS plates with or without antibiotic (tetracycline for Δdps and $\Delta dps \Delta bfr$, cefoxitin for Δbfr) to check the resistance phenotype and determine the ratio of mutant to wild type.

Competitive indices were calculated for each rat by dividing the number of surviving mutants by the number of surviving wild type. This was then divided by the ratio of mutant to wild type in the inoculum. A Student's t-test was performed to compare the differences between the single and double mutants ability to compete. All procedures involving animals followed National Institutes of Health guidelines (93) and

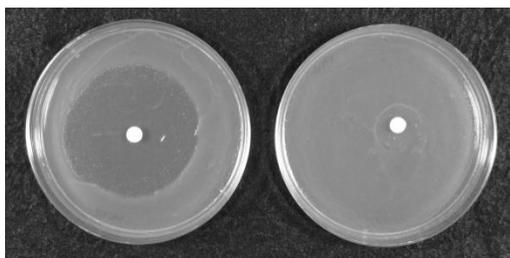
were approved by the Animal Care and Use Committee of East Carolina University. For each bacterial strain two trials of at least 3 animals each were performed.

Results

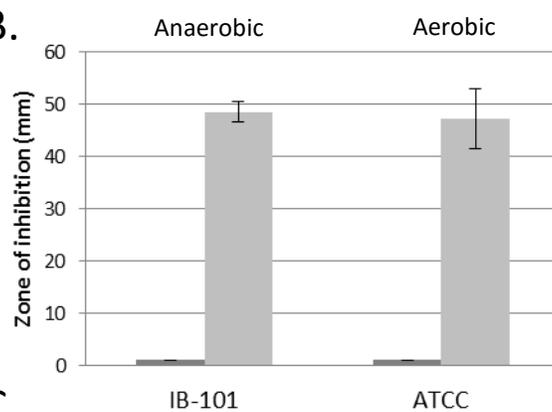
B. fragilis exhibits an oxygen induced resistance to tBOOH. To observe the protective effects of the POST response, disk diffusion assays were used to measure sensitivity to the organic peroxide tBOOH. tBOOH is not easily degraded by cells and can persist allowing for extended periods of oxidative stress and also causes an Fe²⁺ dependent mechanism promoting DNA damage (74, 76-78, 94). *B. fragilis* was very sensitive to tBOOH under anaerobic conditions but when cells were pre-exposed to aerobic conditions for six hours they were completely resistant to tBOOH (Fig. 2.1A). Similar results were seen in assays with two *B. fragilis* strains, IB-101 and ATCC 25285, as shown in the quantified results in Fig. 2.1B. Disk diffusion assays that received six hours of aerobic exposure prior to anaerobic incubation demonstrated no zone of inhibition whereas assays that received no aerobic incubation had a zone of inhibition of about 50 mm. In order to determine a time course for this induced response assays were performed in which we varied the length of time of aerobic exposure. As seen in Fig. 2.1C, complete resistance to tBOOH was only achieved after three hours of aerobic incubation. Interestingly this air induced response requires extended oxygen exposure whereas the rapid peroxide resistance response mediated by OxyR requires less than 30 minutes to mediate protection (68).

Fig 2.1. Sensitivity to tBOOH after oxygen exposure. (A) *B. fragilis* IB-101 was exposed to 55 mM tBOOH either under anaerobic incubation or six hours of aerobic incubation prior to anaerobic incubation. No zone of inhibition is visible in the assays that received aerobic incubation. (B) *B. fragilis* strains IB-101 and ATCC 25285 were exposed to 55 mM tBOOH in a disk diffusion assay and zones of inhibition were measured in mm. Dark grey bars represent cells that were exposed to air for 6 hours prior to anaerobic incubation. Light grey bars represent assays that were not exposed air. (C) Sensitivity of IB-101 exposed to 55 mM tBOOH in disk diffusion assays where time of aerobic incubation was varied. Data represents triplicate assays performed over two independent experiments with average and standard deviation shown.

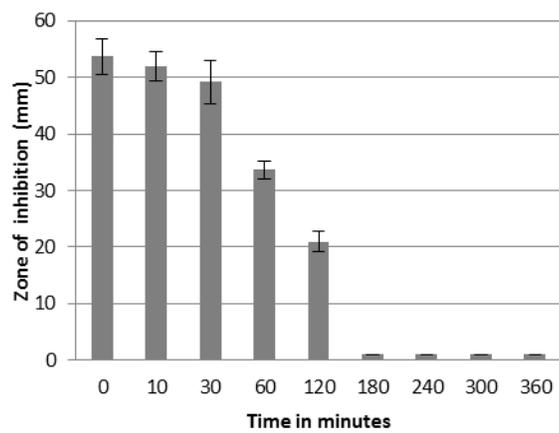
A.



B.

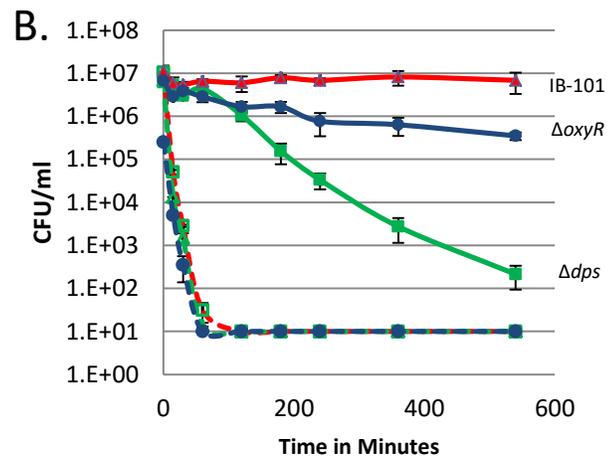
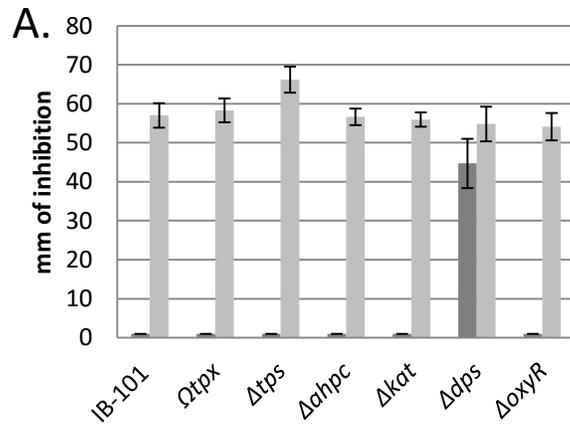


C.



Dps mediates POST resistance to tBOOH. To identify gene products that mediate the increased resistance to tBOOH, disk diffusion assays were performed with known oxidative stress mutants. We first looked at the thiol-peroxidase scavengase (Δtps) and the thioredoxin peroxidase (Ωtpx) because previous studies have shown the mutants to be sensitive to tBOOH under anaerobic conditions (38, 39). Figure 2.2A shows the results from these experiments. Both mutant strains (Δtps and Ωtpx) had complete resistance to tBOOH after incubation in air indicating these do not play a role in the air induced resistance response. We additionally looked at alkyl-hydroperoxide reductase ($\Delta ahpC$) and catalase (Δkat) because of the role they play in the detoxification of peroxides but found that both mutants were similar to wild type after aerobic incubation. Interestingly we found that the Dps mutant (Δdps) had a zone of inhibition of 42 mm even after aerobic incubation. These results indicated that Dps was required for most of the air induced resistance. Previous studies have shown that exposure of *B. fragilis* cells to air generates a rapid induction of *dps* in an OxyR dependent manner (68). However when the $\Delta oxyR$ mutant was screened in the tBOOH disk diffusion assays complete resistance was demonstrated after aerobic incubation. This indicated that Dps mediated the response in an OxyR independent manner suggesting a second regulator of *dps* was responsible for inducing POST *dps* expression.

Fig. 2.2. Dps mediates oxygen induced resistance to tBOOH. (A) Oxidative stress mutants were tested for sensitivity to 55 mM tBOOH in disk diffusion assays. Dark grey bars represent assays exposed to air for 3 hours prior to anaerobic incubation. Light grey bars represent assays that were maintained under anaerobic conditions. (B) Cell viability assays were performed using wild type IB-101 and $\Delta oxyR$ and Δdps mutant strains. Cultures were grown to OD 0.3 and then split. Half of the culture was shaken in air for three hours and the other half was incubated under anaerobic conditions. The cultures were then challenged with 500 μ M tBOOH. Samples were taken over time and CFU/ml was determined. Red (IB-101), blue ($\Delta oxyR$), and green (Δdps) lines represent the data from the aerobically incubated cultures (solid lines) and the anaerobic cultures (dashed lines). Data represents the mean of three biological replicates performed over two independent experiments with standard deviation shown.



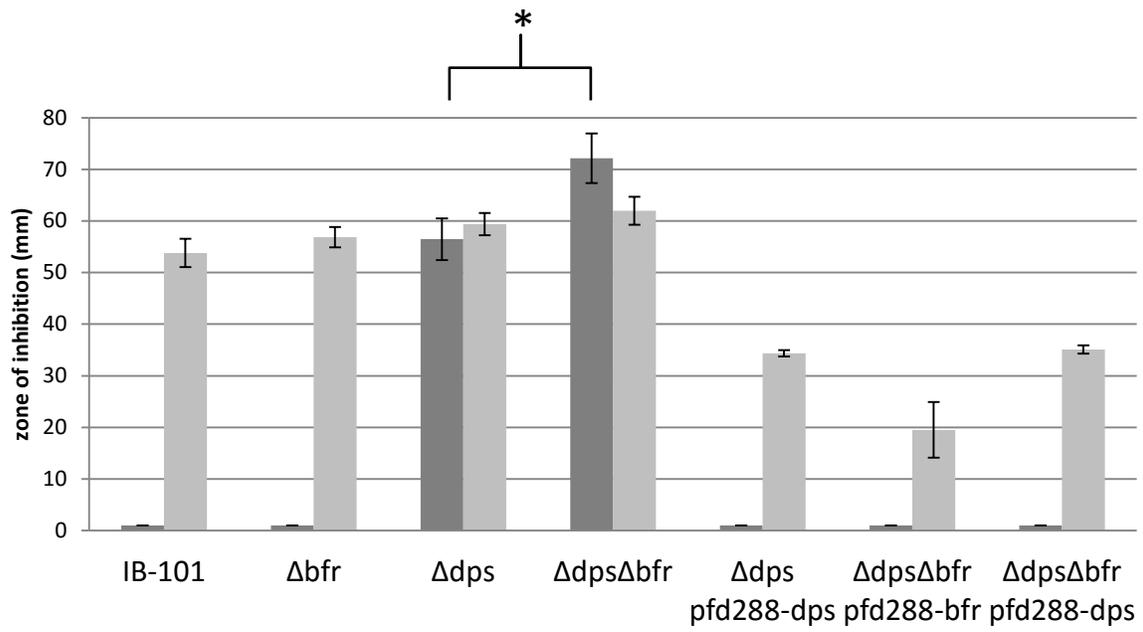
To confirm the results seen in the disk diffusion assays, cell viability assays were performed. Cultures were grown to mid-logarithmic phase and then split. One half was immediately challenged under anaerobic conditions with 500 μ M tBOOH and the number of surviving cells was measured. The other half received aerobic shaking at 37°C for three hours prior to tBOOH challenge. Results in Fig. 2.2B show that IB-101, $\Delta oxyR$, and Δdps demonstrated a rapid loss in cell viability when exposed to tBOOH under anaerobic conditions (dashed lines). However, IB-101 cultures that received three hours of aerobic incubation prior to challenge demonstrated no loss in cell viability. In contrast the Δdps mutant demonstrated a significant, >4 log decrease in cell viability after aerobic induction indicating that Dps is important for this resistance phenotype. Interestingly the $\Delta oxyR$ mutant was more similar to IB-101 and showed a much smaller decrease in cell viability than the Δdps mutant indicating that expression of *dps* was still induced in the $\Delta oxyR$ mutant. Together the data from the disk diffusion assays and the cell viability assays show that Dps is largely responsible for the oxygen induced resistance to tBOOH in an OxyR independent manner.

DpsL contributes to tBOOH resistance. Previous work has demonstrated that the BfDPSL (DpsL) and Dps are similar in protein structure and function (66). Consequently DpsL was investigated to determine if it might account for some of the tBOOH resistance phenotype. Disk diffusion assays were performed with the Δbfr (DpsL) mutant and the results in Fig. 2.3 show that the Δbfr had the same phenotype as wild type. We rationalized that the presence of Dps might be masking the role of DpsL so a $\Delta dps \Delta bfr$ double mutant was constructed. The double $\Delta dps \Delta bfr$ mutant had a greater sensitivity to tBOOH (72 mm of inhibition) than the single Δdps mutant (53 mm

of inhibition). This increased sensitivity to tBOOH also was observed after aerobic incubation suggesting that the absence of both Dps and DpsL causes the cells to be more sensitive to tBOOH.

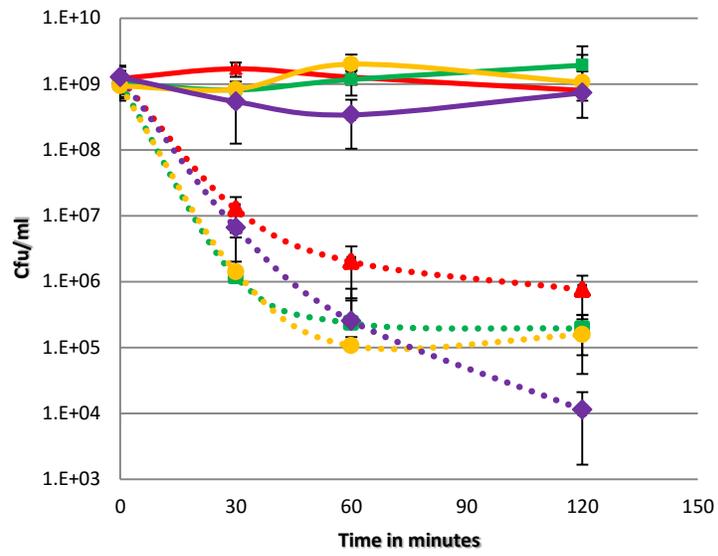
To confirm the roles of Dps and DpsL, the native genes with their native promoters were cloned on a multi-copy plasmid (pFD288) and used to complement the double mutant strain. The *bfr* complemented mutant demonstrated complete protection after aerobic incubation and a significant increase in resistance to tBOOH under anaerobic conditions (Fig. 2.3 and supplemental materials Fig. 2S-1). This was interesting because it demonstrated that *bfr* alone was able to fully protect cells from tBOOH even in the absence of *dps*. Additionally we were able to complement both the single Δdps mutant and the double $\Delta dps \Delta bfr$ mutant with *dps* on pFD288 and restore the oxygen induced resistance response. Together these results indicate that over expression of Dps and DpsL can mediate this oxygen induced resistance to tBOOH.

Fig. 2.3. Dps and DpsL both contribute to tBOOH resistance. Δdps , Δbfr (DpsL), and double $\Delta dps \Delta bfr$ mutants were exposed to 55 mM tBOOH in disk diffusion assays. Dark grey bars represent assays exposed to air for three hours prior to anaerobic incubation and light grey bars are assays that received only anaerobic incubation. Strains were complemented with pFD288 carrying the natural promoter of *dps*(pFD288::*dps*) or *bfr*(pFD288::*bfr*) genes to restore function. Data represents the average of triplicate assays performed over two independent experiments with standard deviation shown. *= P<0.01



Dps and DpsL mediate protection by sequestering iron. In many organisms Dps converts Fe^{2+} to non-reactive Fe^{3+} during periods of oxidative stress to prevent production of highly damaging hydroxyl radicals (50, 56, 79). Similarly it has been shown that the *B. fragilis* DpsL protects against oxidative stress and is structurally very similar to Dps although it contains an iron binding site similar to a bacterioferritin (66). To determine if the oxygen induced response to tBOOH was linked to available reactive iron in the cytoplasm, cell viability assays were performed on cultures treated with dipyrityl, an iron chelator that can enter the cell. As shown in Fig. 2.4, cultures that were treated with dipyrityl (solid lines) did not show a loss in cell viability when exposed to tBOOH whereas cultures that were not treated (dashed lines) showed significant killing. This result demonstrates that the chelation of intracellular iron by dipyrityl rescued the wild type and all mutants suggesting that the mechanism of killing during exposure to tBOOH is linked to the presence of reactive iron. The ability of tBOOH to cause oxidative stress by destruction of iron sulfur clusters and DNA cleavage has been documented (76). Since chelation of iron prevents killing of cells by tBOOH it is likely that the POST response results in the reduction of cytoplasmic iron availability by Dps and DpsL protecting the cells from damage.

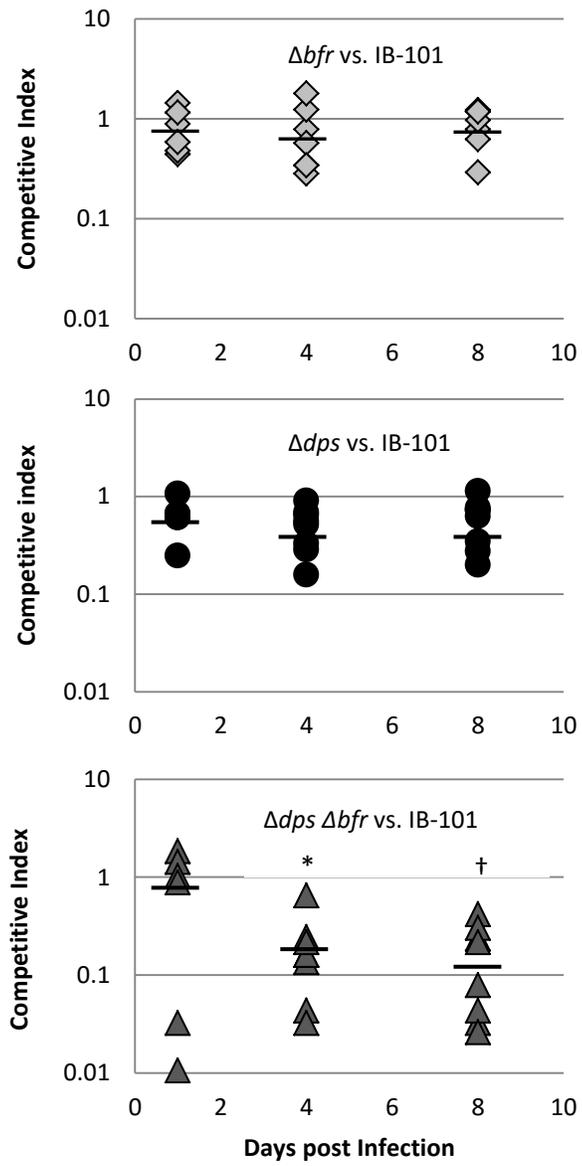
Fig. 2.4. Chelation of iron rescues all strains under anaerobic conditions. Cell viability assays were performed with cultures of IB-101 (Red triangles), Δ dps (green squares), Δ bfr (orange circles), and Δ dps Δ bfr (purple diamonds). Cultures were grown to an OD of 0.3 and then split. Half was treated with 2,2'-dipyridyl (300 μ M) (solid lines) and half was not treated (dashed lines). All cultures were then challenged with 500 μ M tBOOH and CFU/ml was determined over time. Data represents the average of three biological replicates performed over two independent experiments with standard deviation shown.



Dps and DpsL promote survival within the abscess. Factors that contribute to *B. fragilis* survival within the abscess are poorly understood. In a previous study using the rat tissue cage model *in vivo* microarray analysis was used to demonstrate that the *don* locus was highly expressed in the infected tissue cages and was required for maximum survival *in vivo* (27). We reexamined these microarray data and found that there was a 4- to 6-fold increase in expression of *dps* and *bfr* suggesting Dps and DpsL may promote survival within the abscess. Consequently competition assays were performed to determine if the Δdps , Δbfr , and $\Delta dps \Delta bfr$ mutant strains could be out competed by wild type.

Equal numbers of wild type and mutant cells were co-infected into rat tissue cages and samples were taken over a time course. The surviving number of wild type and mutant cells was determined for each sample and a competitive index was calculated. These results are shown in Fig. 2.5 where a competitive index score of 1 indicates that the mutant and wild type compete equally. The mean competitive index scores for Δbfr were 0.82, 0.76, and 0.89 for days 1, 4, and 8 respectively indicating that this mutant was able to compete with wild type. The Δdps mutant showed slight attenuation with mean competitive index scores of 0.64, 0.53, and 0.60 on days 1, 4, and 8. Although there was a decrease in competitive index score the values were not statistically significant. Most interesting was the decreased ability of the double $\Delta dps \Delta bfr$ mutant to compete with wild type. The $\Delta dps \Delta bfr$ mutant had low competitive index scores of 0.21 and 0.17 on days 4 and 8. These competitive index scores were significantly lower than that seen in the single Δdps and Δbfr mutants indicating that loss of both was necessary to significantly affect survival within the abscess.

Fig. 2.5. Dps and DpsL are important for survival *in vivo*. *In vivo* competition assays were performed in the rat tissue cage model. Rat tissue cages were inoculated with equal amounts of WT and either Δdps , Δbfr , or $\Delta dps \Delta bfr$ strains to a final inoculum of 1×10^5 CFU/mL. Samples were taken on days 1, 4, and 8 and plated to determine total CFU/mL. Colonies were then tested for antibiotic resistance phenotypes to determine the ratio of WT to mutant. Competitive indexes were then calculated for each rat using the following formula: output ratio of mutant/WT divided by the input ratio of mutant/WT. Data represents trials of at least three rats from two independent experiments. Mean values for each day are represented by a horizontal line. Student t-tests were performed to compare the single mutant competition assays to the double mutant. * = $P < .05$ was found when the double mutant was compared to either Δdps or Δbfr mutant. † = $P < .01$ was found when the double mutant was compared to either of the single mutants. No difference was seen between Δdps and Δbfr in competition assays.

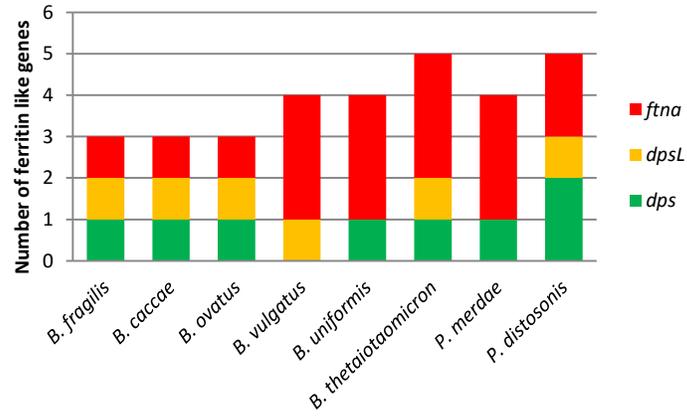


Overall these results indicate that both Dps and DpsL may serve compensatory roles that contribute to survival within the abscess.

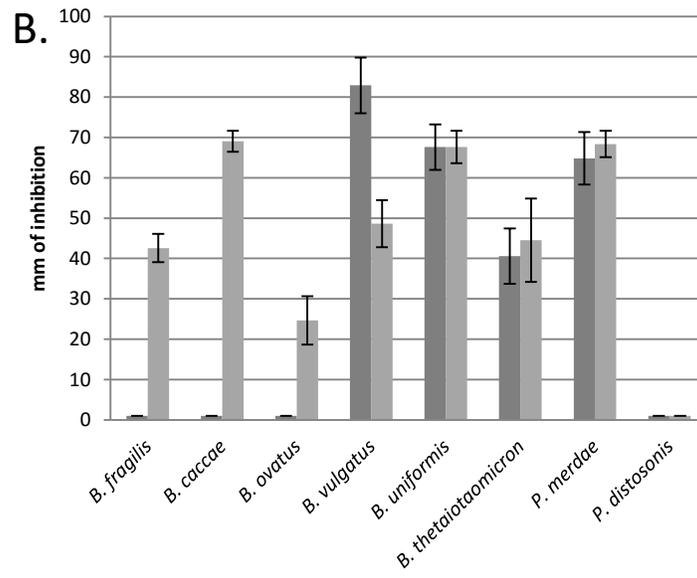
Oxygen induced resistance to tBOOH is not conserved across all members of the *Bacteroides* genus. The *Bacteroides* show large variability in the number of ferritin and ferritin-like proteins coded in their genomes and this diversity is apparent by the many combinations of ferritins, DpsL, and Dps homologues present as shown in Fig. 2.6A (Supplemental materials Table 2S-2) (61). For instance *Bacteroides vulgatus* lacks *dps* but has the genes for DpsL and three ferritins. In contrast, *B. fragilis* contains homologues for Dps, DpsL, and one ferritin and *Bacteroides thetaiotaomicron* has one Dps, one DpsL, and three ferritins. In general the distribution of the genes for these proteins is conserved in each of the *Bacteroides* species. Based on available genome sequences for *B. fragilis* [83 strains], *Bacteroides uniformis* [3], *Parabacteroides merdae* [3], *Parabacteroides distasonis* [8], *Bacteroides caccae* [2], *Bacteroides ovatus* [7], *B. vulgatus* [8], and *B. thetaiotaomicron* [3] we observed that all strains of species possessing *dps* and *dpsL* homologues were consistent. With respect to conservation of the ferritins (*ftnA*), *ftnA1* was conserved in all strains but the presence of *ftnA2* and *ftnA3* was variable in strains of *B. thetaiotaomicron*, *B. uniformis*, and *P. merdae* (40). Because of the great diversity seen in the ferritin family proteins and the different responses to oxidative stress in these organisms we wanted to look at whether the oxygen induced resistance to tBOOH is conserved across the genus.

Fig. 2.6. Oxygen induced resistance to tBOOH is not conserved across the *Bacteroides* genus (A) Graphic representation of the number and type of ferritins (based on sequence homology). Only species that have one *dps*, one *dpsL* and one *ftna* demonstrate the oxygen induced resistance to tBOOH. (B) Disk diffusion assays were performed with 55mM tBOOH with closely related strains of *Bacteroides* and *Parabacteroides*. Dark grey bars represent assays exposed to air for 3 hours prior to anaerobic incubation. Light grey bars represent assays that were not exposed to aerobic incubation. Zones of inhibition were measured and reported above.

A.



B.



Disk diffusion assays were performed using several members of the *Bacteroides* genus with results shown in Fig. 2.6B. Interestingly the species could be grouped based on the number and type of ferritin homologues found in each genome. *Bacteroides caccae* and *Bacteroides ovatus* were similar to *B. fragilis* containing the same number and type of ferritin homologues (1 Dps, 1 DpsL, and 1 ferritin) and they all demonstrated the same phenotype in the tBOOH sensitivity assays. None of the other species tested demonstrated the aerobic induced resistance to tBOOH. *B. vulgatus*, which has no Dps homologue showed greater sensitivity to tBOOH after oxygen exposure than under anaerobic conditions. Additionally *B. uniformis*, *B. thetaiotaomicron*, and *P. merdae* showed little to no difference in resistance levels regardless of aerobic incubation indicating these species do not have the oxygen induced resistance response. Most noteworthy was the complete resistance to tBOOH seen in *P. distasonis* under both conditions.

When comparing the number and type of ferritins in each species and their resistance profiles some interesting trends can be seen. The first is that only species that contain one Dps, DpsL, and ferritin homologue have the oxygen induced resistance response phenotype. Interestingly, *B. vulgatus* which lacks Dps had high sensitivity to tBOOH after aerobic exposure whereas *P. distasonis* which has two Dps homologues, was completely resistant to tBOOH supporting the idea that Dps plays a central role in resistance to tBOOH. However, *B. uniformis*, *B. thetaiotaomicron*, and *P. merdae*, which do have a *dps* homologue did not show the inducible resistance phenotype. One explanation for this is that these species have evolved different regulatory mechanisms for *dps* and *dpsL* expression.

Discussion

B. fragilis has an extensive network of iron storage proteins in the ferritin superfamily, Dps, DpsL, and ferritin, all of which are linked in some way to the oxidative stress response (28, 63, 68). The current report is focused on the role of Dps and DpsL in protection against extended exposure to oxidative stress as part of the POST response. The assay used for this work required a period of prolonged aerobic incubation to rescue cells from tBOOH killing and the results showed that Dps and to a lesser extent DpsL both contributed to protection (Fig. 2.3). Support for this is that the Δdps mutant was extremely sensitive to tBOOH and could not be rescued by aerobic induction. In addition, the double $\Delta dps \Delta bfr$ mutant had greater sensitivity to tBOOH than the single mutants indicating that Dps and DpsL function in a similar manner to protect against this stress. Complementation of the $\Delta dps \Delta bfr$ mutant with either *bfr* or *dps* resulted in complete resistance to tBOOH after oxygen exposure and provided greatly enhanced resistance under anaerobic conditions (Fig. 2.3). Finally the Δdps , Δbfr , and $\Delta dps \Delta bfr$ mutants were equally protected from tBOOH killing by the iron chelating agent dipyriddy indicating that the protective mechanism provided by both of these proteins involves removal of reduced iron from the cytoplasm during periods of oxidative stress (Fig. 2.4). Taken together these data are evidence that during the POST response induction of Dps and DpsL protects cells from damage caused by cytoplasmic ferrous iron. Given that Dps and DpsL share similar functional properties and either can rescue the POST phenotype it appears that it is differential regulation of the *dps* and *bfr* genes that is key to understanding their varied contributions to protection.

The acute oxidative stress response is designed to rapidly detoxify and minimize the effects of a sudden exposure to oxidative stress. This occurs within minutes of exposure and OxyR is the major regulator for this response (15). By comparison, previous work and the findings from this paper show that the POST response is a more global shift in cellular regulation and physiology occurring after exposure to air for greater than 1 hour (13). Analysis of *dps* regulation in *B. fragilis* has shown that transcription is rapidly induced by exposure to either H₂O₂ or air during exponential growth (15). This is mediated by OxyR and is considered to be part of the acute oxidative stress response. However, as demonstrated by the studies in this report, as well as microarray analysis of gene transcription during prolonged air exposure, Dps also plays an important role in the POST response and this regulation is independent of OxyR (13). Most interesting was that prolonged exposure to air was required for protection from tBOOH. In exponentially growing cells OxyR rapidly induces *dps* expression during oxidative stress, however as shown in *E. coli*, OxyR does not induce expression of *dps* during stationary phase growth even when cells are exposed to hydrogen peroxide (95). This suggests that the POST response requires the second regulator to induce expression of *dps* because OxyR does not function in the non-growing cells which is similar to how *E. coli* regulates *dps* expression during various growth phases (50, 95). In contrast, *bfr* gene expression is relatively insensitive to H₂O₂ but it is strongly induced by exposure to air for greater than 1 hour or in anaerobic stationary phase cultures (28, 66). Overall these data demonstrate a role for Dps in both the acute and POST oxidative stress responses whereas DpsL appears to only have a role in the POST response. The regulation of the POST response is of great

interest because it leads to protection when cells are not rapidly growing and allows for the high aerotolerance seen in *B. fragilis*. Dps and DpsL both have roles during prolonged oxidative stress and further studies of their differential regulation should help identify the important POST regulator(s).

To investigate the roles of Dps and DpsL in survival *in vivo*, growth of wild type and mutant strains were compared in a rat tissue cage model of infection. This model has effectively been used to show the attenuation of mutant strains of *B. fragilis* within an artificial abscess (26, 27). Experiments that compared the ability of the wild type strain to outcompete single Δdps and Δbfr mutants showed only slight attenuation that was not statistically significant. However the double $\Delta dps \Delta bfr$ mutant was significantly attenuated, as shown in Fig. 2.5, indicating that Dps and DpsL are both required for maximum survival in the abscess model. These data also indicate that Dps and DpsL may be playing overlapping roles in protecting the cells from oxidative stress damage *in vivo* because the absence of both was required to see the phenotype. As previously shown in this model, *B. fragilis* reaches high cell numbers and then enters into a stationary-like phase where that high cell density is maintained (26, 27). Interestingly, on day 1 the double $\Delta dps \Delta bfr$ mutant was able to compete effectively with wild type and was close to 50% of the 10^8 - 10^9 CFU/ml, however on day 4 and 8 there was a decrease in the competitive ability of the double mutant as seen in Fig. 2.5. Results from the *in vitro* growth analysis (Supplemental materials Fig. 2-S2) indicate there is no general growth defect in the double mutant. It is reasonable to suggest from these data that the double mutant may be experiencing DNA and protein damage due to oxidative stress and higher levels of ferrous iron within the cytoplasm during days 4-8.

Oxidative stress occurs immediately upon bacterial translocation from the anaerobic intestinal tract to the oxygenated peritoneal cavity with additional stress resulting from the immune systems response to bacterial presence in the peritoneum making high levels of ferrous iron toxic to cells. To survive, *B. fragilis* requires an effective system for management of intracellular iron which in part is provided for by Dps and DpsL. The contribution of Dps and other ferritins to virulence has been shown in other organisms. Mutations in ferritin family genes in *Salmonella enterica*, *Haemophilus influenzae*, and *Streptococcus pyogenes* were responsible for defects in survival *in vivo* and effected susceptibility to killing from oxidative stress (96-99). These results are similar to those seen here with *B. fragilis*. Though the majority of the *Bacteroides* have *dps* and *bfr* (DpsL), only three species, *B. fragilis*, *B. caccae*, and *B. ovatus* demonstrate the air inducible tBOOH resistance phenotype suggesting that the regulation of these genes may provide some advantage in an extraintestinal site (Fig. 2.6).

The normal environment for *B. fragilis* is the large intestine which is known to be highly anaerobic yet *B. fragilis* has a robust oxidative stress response. It has been shown that resistance to oxidative stress is important for establishment of intra-abdominal abscesses but this habitat is a dead end leaving the question of what selects for resistance to oxidative stress in the colon. One thought is that this stress may occur during the inflammatory response. Inflammation of the intestinal tract caused by *Campylobacter jejuni*, *Helicobacter pylori*, and many other pathogens results in increased levels of oxidative stress within the epithelial layer and the intestinal tract (100-103). *B. fragilis* is closely associated with the intestinal epithelium which has been

shown to receive significant DNA damage from reactive oxygen species from the host immune response (9, 101, 104). Additionally it has been shown that an oxygen concentration gradient exists extending out from the epithelial surface so that *B. fragilis* may be exposed to an environment with as much as 8% oxygen depending on precise site of colonization (8, 9). It is reasonable to suggest that these conditions could cause significant oxidative stress to the organism. Being able to store and scavenge reactive ferrous iron would be essential for survival of the *Bacteroides* in this changing environment and during the inflammatory response. Therefore the diversity and quantity of ferritin-like proteins used by *B. fragilis* would give it an advantage and promote survival during these times. Then in the event of intestinal damage and the translocation of the natural flora into the peritoneal cavity, those organisms that are better suited to survive the oxidative burst of the immune response will be able to persist promoting the establishment of an abscess. This may in part explain why *B. fragilis* is so frequently isolated from intra-abdominal abscesses. Additionally, transmission of *B. fragilis* from mother to child results in exposure to an aerobic environment and an effective oxidative stress response would provide for more efficient transmission (1, 105).

This report demonstrates that Dps and DpsL are part of the POST response in *B. fragilis*. These proteins are responsible for storing and preventing ferrous iron from producing hydroxyl radicals in the cytoplasm during periods of oxidative stress. Studies are needed to further elucidate the regulation of the POST response and transcriptional control of *dps* and *bfr*. Dps and DpsL provide protection for the cells during survival within the abscess and ultimately within the intestinal tract. Overall these data indicate that *B. fragilis* and potentially other members of the *Bacteroides* must be able to

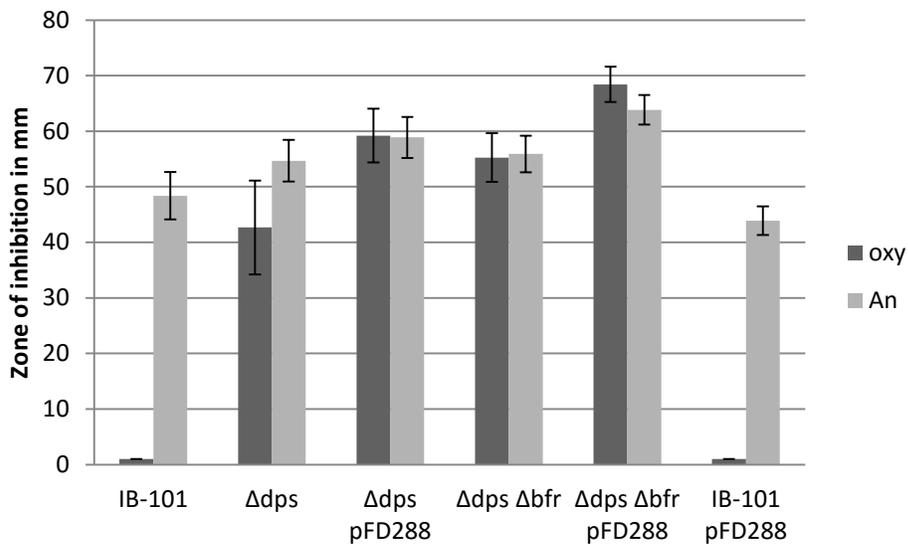
efficiently manage iron in order to survive as members of the natural flora of the intestinal tract.

Acknowledgements

We would like to thank Anita Parker, Greg Wells, and Ivan Ndamukong for advice and technical assistance throughout this work. This work was supported by PHS Grant AI40588 (to C.J.S).

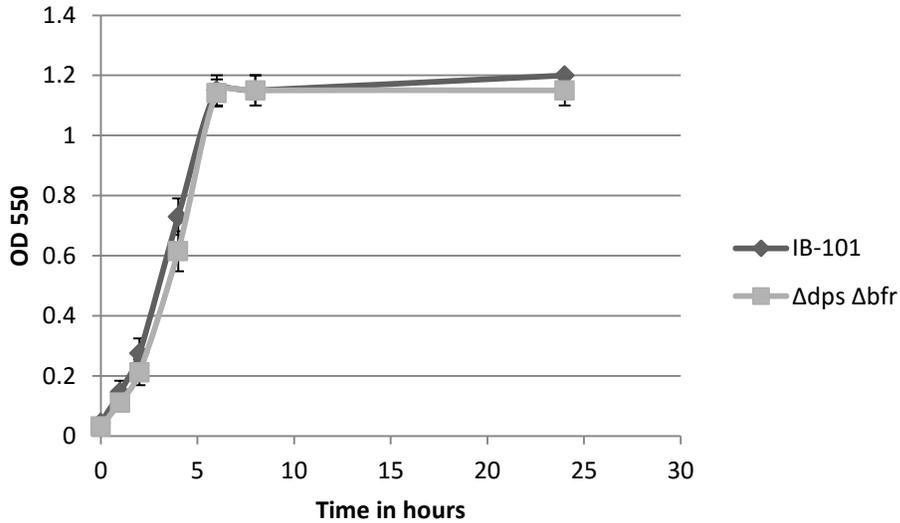
Supplemental section

Supplemental Fig. 2S-1 Additional Disk diffusion assay controls



Supplemental Fig. 2S-1: Disk diffusion assays were performed as described in the Materials & Methods. In brief, plates were inoculated and then challenged with tBOOH filter disks. Assays were kept either under anaerobic conditions (light grey bars) or given three hours of aerobic incubation prior to anaerobic overnight incubation. Zones of inhibition were measured in mm. This figure is a control that demonstrates the empty vector, pFD288, does not complement the Δ dps and Δ dps Δ bfr mutants.

Supplemental Figure 2S-2: Growth curve of *Δdps Δbfr*



Supplemental Figure 2S-2: The double *Δdps Δbfr* mutant does not have a general growth defect. Cultures were subcultured from overnight stationary phase cultures and growth was measured for IB-101 and *Δdps Δbfr* in BHIS under anaerobic conditions. Triplicate cultures for each strain were followed over two independent experiments. Averages of the six replicates are reported with standard deviation shown. Results indicate that the double mutant does not have a growth defect when compared to WT under normal anaerobic growth conditions.

Supplemental table 2S-1 Primers used in this study

| Primer | 5'-3' Sequence | Purpose |
|-------------------------|---------------------------------------|---|
| BfrsL-F | CAGTGGATCCCCTAAACCAAAAGAAATTATGG C | Amplification of full length <i>bfr</i> gene used in complementation forward Primer |
| BfrsL-R | CAGTGAGCTCTGGGGTATTTCCCTCTTTCTA | Amplification of full length <i>bfr</i> gene used in complementation reverse primer |
| Bfr-1-For | CCCCATGTTACATAACC | Used to verify deletion of <i>bfr</i> gene in $\Delta dps\Delta bfr$ forward primer |
| Bfr-2-Rev | CGGTCACTGTAGCAAAGCG | Used to verify deletion of <i>bfr</i> gene in $\Delta dps\Delta bfr$ reverse primer |
| AHPC-1- <i>EcoRI</i> | GCCATCAGAAATTCCTCCCATC | Amplification of N-terminal region of <i>ahpc</i> for deletion construct forward primer |
| AHPC-2- <i>EcoRI</i> | CCTGTACTTTGAATTTCAGGC | Amplification of N-terminal region of <i>ahpc</i> for deletion construct reverse primer |
| AHPC-3- <i>BglII</i> | GGTCGGTAAGATCTAAACAGC | Amplification of C-terminal region of <i>ahpc</i> for deletion construct forward primer |
| AHPC-4- <i>SphI</i> | CCTTTCCAGCATGCTCTATC | Amplification of c-terminal region of <i>ahpc</i> for deletion construct reverse primer |

Supplemental Table 2S-2: Gene accession numbers for Ferritin homologs in the *Bacteroides*

| Strains | <i>dps</i> genes | GenBank Accession # | <i>dpsL/bfr</i> genes | GeneBank Accession # | <i>ftnA</i> genes | GeneBank Accession # |
|---------------------------------------|------------------|----------------------|-----------------------|----------------------|-------------------|----------------------------------|
| <i>B. fragilis</i> 638R | 1 | AAG02618 | 1 | CBW23774 | 1 | AAK29742 |
| <i>B. caccae</i> ATCC 43185 | 1 | EDM20674 | 1 | EDM19603 | 1 | EDM20344 |
| <i>B. ovatus</i> ATCC 8433 | 1 | EDO09702 | 1 | EDO11031 | 1 | EDO14102 |
| <i>B. vulgatus</i> ATCC 8482 | None | - | 1 | ABR41194 | 3 | ABR41141 ABR40404 ABR41143 |
| <i>B. uniformis</i> ATCC 8492 | 1 | EDO55385 | None | - | 3 | EDO51842 EDO55572 EDO55570 |
| <i>B. thetaiotaomicron</i> ATCC 29148 | 1 | AAO79820 | 1 | AAO78928 | 3 | AAO76480 AAO76214 AAO76216 |
| <i>P. merdae</i> ATCC 43184 | 1 | EDN86811 | None | - | 3 | EDN87770 EDN84345 EDN84347 |
| <i>P. distasonis</i> ATCC 8503 | 2 | ABR42394 ABR42396 | 1 | ABR43121 | 2 | ABR41895 ABR42042 |

Table 2S-3: Strains used in supplemental section

| Strain or plasmid | Phenotype and/or genotype ^a | Reference or source |
|--------------------|---|---------------------|
| <i>Bacteroides</i> | | |
| strains | | |
| IB-101 | <i>B. fragilis</i> 638R Clinical Isolate, Rif ^r | (84) |
| IB 336 | IB-101 Δ <i>dps::tetQ</i> , Rif ^r Tet ^r | (63) |
| IB-542 | IB-336 Δ <i>bfr::cfx</i> , Rif ^r Tet ^r Cfx ^r | This Study |
| IB-574 | IB-336 pFD288, Rif ^r , Tet ^r , Cfx ^r Erm ^r | This Study |
| IB-575 | IB-542 pFD288, Rif ^r , Tet ^r , Erm ^r | This Study |
| IB-579 | IB-101 pFD288, Rif ^r , Tet ^r , Erm ^r | |
| Plasmids | | |
| pFD288 | (Sp ^r),Erm ^r , oriT, pUC19:: <i>pBI143</i> 8.8-kb shuttle vector | |

^aErm^r, erythromycin resistance; Cfx^r, cefoxitin resistance; Rif^r rifampicin resistance; Tet^r, tetracycline resistance; Sp^r, spectinomycin resistance. For *Bacteroides-E. coli* shuttle vectors, parentheses indicate antibiotic resistance expression in *E. coli*.

CHAPTER THREE: STRATEGIES TO IDENTIFY A SECOND REGULATOR OF DPS

Rationale:

The findings in Chapter 2 demonstrated an important role for a second regulator of *dps* expression. This was shown by the $\Delta oxyR$ mutant being fully resistant to tBOOH after prolonged exposure to air Fig. 2.2. This result was surprising because previous work had shown that OxyR rapidly induced the expression of *dps* in response to oxygen and hydrogen peroxide (68). These data indicated that a second regulator was able to induce expression of *dps* during exposure to oxygen that was independent of OxyR. A second regulator in *dps* expression was suggested by expression data from microarray data from cultures receiving extended aerobic exposure (28). These results shaped the idea that the unidentified second regulator was important in mediating protection from extended oxygen exposure during the POST response. Characterization of this regulator will provide significant insight into the control and understanding of the POST response.

Introduction

The induction and repression of ferritin genes is complex and typically will involve several regulators in order to accomplish effective regulation. There are three conditions which lead to the induction of ferritin genes. Under conditions of excess iron, ferritins will be induced in order to prevent the accumulation of high levels of ferrous iron within

cells. A second condition is oxidative stress which has been previously discussed. The third condition that causes induction of ferritin genes occurs when cells enter into stationary phase. Under all three conditions the presence of excess ferrous iron is dangerous for the cell and thus the cells have developed many strategies to control these genes in a coordinated manner and reduce the levels of ferrous iron.

The expression of *dps* has been best studied in *E. coli* and has been shown to be multi-leveled. Expression of *dps* in *E. coli* occurs under oxidative stress conditions as well as during stationary phase. However, the expression of *dps* is managed by several different regulators. During oxidative stress, OxyR becomes active and recruits σ^{70} resulting in a rapid induction of *dps* expression thus protecting the cell from hydroxyl radical production and DNA damage (50). When the cells begin to enter into stationary phase, the stationary phase sigma factor σ^S controls the expression of *dps*. In contrast, during logarithmic growth the nucleoid-associated proteins Fis and H-NS work in conjunction to repress the expression of *dps* under logarithmic growth (50, 106). It has been shown that H-NS binds to the *dps* promoter and prevents transcription initiation by the housekeeping sigma factor, σ^{70} . However this repression can be overcome by σ^S which is induced when the cells begin to enter stationary phase resulting in the increased expression of *dps*. Additionally Fis forms a complex with σ^{70} which is able to block transcription by σ^S thus preventing *dps* expression when Fis is present. As cells enter into stationary phase, the levels of Fis decrease and will eventually free the σ^S promoter sequence (50, 106, 107). Therefore the activities of these two repressors keep the expression levels of *dps* very low during logarithmic growth unless the cell experiences oxidative stress at which point OxyR is able to drive expression. H-NS and

Fis in conjunction with OxyR and σ^S limit the expression of *dps* in *E. coli* to conditions of oxidative stress and during stationary phase.

The organisms *Bacillus subtilis* and *Streptococcus pneumoniae* utilize the peroxide resistance regulator PerR to regulate the expression of their *dps* homologues (*mrgA* and *dpr* respectively) (81). PerR is a member of the ferric uptake regulator (Fur) family of metallo-regulators and utilizes Fe^{2+} to sense and respond to peroxide stress (108, 109). This protein family functions as repressors which in the presence of their respective metal ligand prevent the expression of genes by binding and blocking transcription. In the case of the ferrous iron uptake regulator Fur, when levels of Fe^{2+} are high, Fur binds to Fe^{2+} and represses genes associated with iron uptake (108). This serves to prevent the accumulation of excess transition metals in the cytoplasm. PerR also binds ferrous iron however the iron binding cleft, unlike other members of the Fur family, is sensitive to oxidation in the presence of peroxides. In the event of oxidative stress the resulting hydrogen peroxide will catalyze a Fenton reaction with the iron bound in the cleft resulting in oxidation of conserved histidine residues, resulting in the release of iron and the loss of gene repression (108). The coordinated activity of Fur and Per will result in the expression of genes such as *mrgA* and *dpr* and repression of iron acquisition systems in response to excess iron and oxidative stress.

Given the diversity of candidate regulators found in *B. fragilis*, a variety of approaches were undertaken to identify the second regulator of *dps* expression. The first was to investigate and define the regulons of two potential candidate regulators that fall into the Fur family of regulators. *B. fragilis* has three Fur-like regulators FurA, B, and C. Based on homology the three Fur-like regulators appear to fit into the three known

classes of Fur family regulators and were thus assigned as follows: FurA as Fur, FurB as PerR, and FurC as Zur. Given the role that PerR plays in the regulation of *dps* expression in Gram positive organisms it was given a high priority for investigation as the second regulator of *dps*.

Another possible second regulator would be a stationary phase sigma factor homologue. No stationary phase sigma factor has been identified in *B. fragilis* but there are many ECF- family sigma factors that could act cooperatively to manage gene expression during stationary phase. Of these, there are 14 that are affected by aerobic exposure (71). Given that expression of these sigma factors is affected by oxygen exposure and that the resistance to tBOOH is also mediated by extended aerobic exposure, these regulators are worth investigating as potential second regulators of *dps* expression. Of particular interest are EcfO and SigOF because of the roles these regulators have shown in response to oxidative stress.

This chapter is focused on a series of experiments performed in the attempt to identify the second regulator of *dps*. The genetic regulation of *dps* in other organisms is multifaceted and that appears to be true for *B. fragilis* as well. The work in this chapter provides strong evidence that SigOF is the second regulator of *dps* and describes *dps* expression patterns consistent with POST phase induction of *dps*. We have also gained a great deal of information about the regulatory activities of the *B. fragilis* Per and Fur homologues providing some understanding into the role these two regulators are playing. The data show that regulation of *dps* expression during the POST response is important for survival and therefore the identification of the second regulator provides

information into the different cellular processes that allow for the tolerance of extended aerobic conditions.

Methods and materials

Bacterial strains and growth conditions. *Bacteroides* strains used in this study are listed in Table 3.1. All strains were grown anaerobically in brain heart infusion broth supplemented with hemin, cysteine, and NaHCO₃ (BHIS) unless otherwise noted (83). Rifampicin (20µg/ml), gentamicin (50µg/ml), tetracycline (5µg/ml), ceftiofur (25µg/ml), and erythromycin (10µg/ml) were added to the media when needed.

Construction of mutant strains. Primer sequences used for genetic manipulations are listed in Table 3.2. Briefly, the $\Delta sigOF Tet^r$ mutant was constructed by PCR amplification of the N-terminal fragment of sigOF using oligonucleotides containing EcoRI recognition site at the 5' end and BamHI site at the 3' end. The fragment was cloned into a suicide vector, pFD516. A C-terminal fragment was amplified using the same approach except oligonucleotides contained a BamHI recognition site at the 5' end and PstI at the 3' end. This was then cloned into pFD516 containing the N-terminal fragment. A 2.2kb tetracycline cassette (*tetQ*) was inserted in between the N and C-terminal fragments using the BamHI site creating pFD1090. This plasmid was then mobilized into IB-101 generating IB-478 $\Delta sigOF::tetQ$. The $\Delta sigOF::cfx$ (IB-577) mutant was constructed by modifying pFD1090 (plasmid used to construct $\Delta sigOF::tetQ$) by removing the *tetQ* cassette by restriction digest with BamHI. The 1.3kb *cfx* cassette was then PCR amplified from pFD351 with flanking BamHI restriction enzyme sites. The *cfx* cassette was then cloned into pFD1090 creating pFD1250. This plasmid was then

mobilized into *B. fragilis* IB-101 and exconjugants were selected on BHIS plates containing rifampicin, gentamicin, and cefoxitin (91). Sensitivity to erythromycin and PCR were used to confirm the double-crossover allelic exchange of *sigOF::cfx* mutation in strain IB-577.

Construction of the double $\Delta sigOF \Delta dps$ mutant, IB-580, was performed by mobilizing pFD786 (Δdps mutational construct) into IB-577. Mutants were selected on rifampicin, gentamicin, and tetracycline. PCR was performed to confirm the double-cross over allelic exchange of *dps::tetQ*. Construction of the double $\Delta sigOF \Delta oxyR$ IB-578 mutant was performed in a similar manner where the pFD-754 ($\Delta oxyR$ mutational construct) was mobilized into IB-577. Mutants were selected on rifampicin, gentamicin, and tetracycline. PCR was performed to confirm the double-cross over allelic exchange of *oxyR::tetQ*.

Table 3.1: Strain table. Strains used in the experiments outlined in Chapter 3.

| Strain or plasmid | Phenotype and/or genotype ^a | Reference or source |
|--------------------|---|---------------------|
| <i>Bacteroides</i> | | |
| strains | | |
| IB-101 | <i>B. fragilis</i> 638R Clinical Isolate, Rif ^r | (84) |
| IB 298 | IB-101 $\Delta oxyR::tetQ$, Rif ^r Tet ^r | (68) |
| IB 336 | IB-101 $\Delta dps::tetQ$, Rif ^r Tet ^r | (63) |
| IB-368 | IB-101 $\Delta per::cfx$ Rif ^r Cef ^r | |
| IB-542 | IB-336 $\Delta bfr::cfx$, Rif ^r Tet ^r Cfx ^r | This Study |
| IB-577 | IB-101 $\Delta sigOF::cfx$ Rif ^r , Cfx ^r | This Study |
| IB-578 | IB-577 $\Delta oxyR::tetQ$ Rif ^r , Tet ^r , Cfx ^r | This Study |
| IB-580 | IB-577 $\Delta dps::tetQ$ Rif ^r , Tet ^r , Cfx ^r | |
| BER-2 | IB-101 $\Delta fur::tetQ$ Rif ^r Tet ^r | |
| BER-74 | IB-101 $\Delta bfr::cfx$ Rif ^r , Tet ^r | (66) |
| <i>E. coli</i> | | |
| strains | | |
| DH10B | F ⁻ mcrA $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi 80lacZ\Delta M15$ $\Delta lacX74$ recA1 endA1 araD139 $\Delta(ara leu)$ 7697 galU galK rpsL nupG λ - | Invitrogen |
| HB101:RK2 31 | HB-101 containing RK231, Km ^r Tc ^r St ^r | (89) |
| Plasmids | | |
| pFD516 | <i>Bacteroides</i> suicide vector derived by deletion of <i>Bacteroides</i> replicon pBI143 from pFD288, (Sp ^r), Erm ^r | (90) |
| pFD754 | 2.2kb <i>tetQ</i> cassette was inserted into NdeI/SalI sites of pFD750 to generate $\Delta oxyR::tetQ$. | (69) |
| pFD786 | A fragment of the <i>dps</i> gene was removed from pFD760 at BamHI and MscI and replaced with <i>tetQ</i> to generate $\Delta dps::tetQ$ | (63) |
| pFD1090 | A deletion of the <i>sigOF</i> gene cloned into EcoRI/PstI sites of pFD516 with a 2.2kb <i>tetQ</i> cassette inserted in the BamHI site. | |
| pFD1250 | A 2.2kb <i>tetQ</i> cassette was removed from pFD1090 using BamHI and replaced with a 1.4kb <i>cfx</i> cassette. | |

^aErm^r, erythromycin resistance; Cfx^r, cefoxitin resistance; Rif^r rifampicin resistance; Tet^r, tetracycline resistance; Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance. For *Bacteroides-E. coli* shuttle vectors, parentheses indicate antibiotic resistance expression in *E. coli*.

Table 3.2 Table of all primers used in studies outlined in Chapter 3

Table 3.2 Primers used in this study

| Primer | 5'-3' Sequence | Purpose |
|---------------|----------------------------------|--|
| Nterm-eco | acgtgaattcttcggagcctcttctaaattgg | Forward primer for amplification of the N-terminal portion of the <i>ΔsigOF</i> mutant |
| Nterm-bam | acgtggatccggtgccggttttaagcgggtaa | Reverse primer for amplification of the N-terminal portion of the <i>ΔsigOF</i> mutant |
| C-term-bam | acgtggatccggaactgaaggactttgtttg | Forward primer for amplification of the C-terminal portion of the <i>ΔsigOF</i> mutant |
| C-term-pst | acgtctgcagaattgtcctaacaatggcagg | Reverse primer for amplification of the C-terminal portion of the <i>ΔsigOF</i> mutant |
| cfx-F BamHI | gtcgactctagaggatcccc | Amplify cefoxitin cassette with flanking BamHI sites |
| cfx-R BamHI | gactggatccgccgaacaggaagaagaaa | Amplify cefoxitin cassette with flanking BamHI sites |

Disk Diffusion Assays. Disk diffusion assays were performed as previously described (66). In brief, 100 μ l of overnight culture was spread on BHIS plates (without cysteine) and a 6-mm filter disk was placed in the center of the plate. The disks were then saturated with 10 μ l of 55 mM tBOOH unless otherwise noted. Disk diffusion assays were also exposed to 10% H₂O₂ where noted. Plates either were immediately incubated anaerobically at 37°C or they received 3 hours of aerobic incubation at 37°C prior to anaerobic incubation. Following overnight anaerobic incubation, the diameters of the zones of growth inhibition were measured, and the results were reported as the average of at least two independent experiments performed in triplicate. A Student's two tailed *t*-test was performed to determine significant differences between populations when appropriate.

Growth conditions for microarray and qRT-PCR analysis. All cultures were grown to an OD₅₅₀ of 0.4-0.5. For microarray analysis to determine the PerR regulon WT IB-101 and the Δ *perR* mutant (IB-368) cultures were grown to an OD₅₅₀ of 0.5 and then split. Half the culture was shaken aerobically for 3 hours at 37°C. The remaining half was treated with chloramphenicol and harvested immediately by centrifugation. After three hours of aerobic exposure the culture was harvested as described above. Analysis of the Fur regulon under iron deplete conditions was performed as follows. IB-101 and the Δ *fur* mutant (BER-2) were grown in minimal media supplemented with 5 μ g/mL Protoporphyrin IX to an OD₅₅₀ of 0.4 (63). Additionally IB-101 and the Δ *furA* mutant were grown in iron deplete conditions. The iron depletion media was prepared from minimal media treated with 40 μ M of 2,2'-dipyridyl an iron chelating agent. Cells were then grown to an OD₅₅₀ of 0.4 and harvested as previously described. To

investigate the levels of *dps* in the double $\Delta sigOF \Delta oxyR$ mutant cultures of WT, $\Delta oxyR$, $\Delta sigOF$ and $\Delta sigOF \Delta oxyR$ were grown to an OD₅₅₀ of 0.5 and then split. Half received 1 hour of aerobic shaking at 37°C while the other half was treated with chloramphenicol and harvested. After 1 hour of aerobic exposure the remaining culture was harvested. RNA was isolated for these cultures as described below.

RNA isolation and qRT-PCR analysis. RNA isolation was done by the hot-phenol method as previously described (42, 71). The samples were treated three times with Turbo DNA-free DNase (Ambion/Life technologies Inc.) and purified by phenol chloroform extraction. Twenty micrograms of DNA was used for first strand cDNA synthesis in a reaction mixture with 13ng per μ L random hexamers, 0.5mM deoxynucleotide triphosphates (dNTPs), 1 X first-strand buffer, and 1 μ L Superscript II RNase-H-reverse transcriptase I (Invitrogen, Carlsbad, CA). For quantitative reverse transcriptase PCR (qRT-PCR) the reaction mixture contained 12.5 μ L of 2 X iQ SyBR green Supermix, 1.5 μ L of 5mM forward primer, 1.5 μ L of 5mM reverse primer, 4.5 μ L H₂O, and 5 μ L of cDNA template (diluted to 2 ng per μ L) per well. All samples were run in duplicate from at least two biological replicates. Relative expression level was determined by the Pfaffl method (110) using 16S RNA as a reference.

Microarray analysis. For microarray expression analysis, single stranded cDNA was converted to double stranded cDNA as previously described (71). Double-stranded cDNA was synthesized with the Super Script^R Double-Stranded cDNA synthesis kit (Life technologies, Inc.). One microgram of double stranded cDNA was labeled with cy3 and hybridized to microarray slides, and processed by the Florida State University

Roche/NimbleGen Microarray Facility. For each experimental condition at least two independent trials were performed. Each trial consisted of a high-density oligonucleotide whole-genome expression microarray (Roche/NimbleGen) with eight technical replicates of each probe per slide. The raw microarray expression data was normalized together by using the robust multiarray average (RMA) algorithm, as implemented in Roche Deva 1.1 software. The normalized data were analyzed by using ArrayStar software (Dnastar, Inc., Madison, WI). Putative regulons were determined for Fur and PerR by comparing genes that were highly expressed (≥ 5 fold) in the mutant strain but not in Wild type. A significant increase in gene expression would be expected in the mutant strains due to absence of the Fur and Per repressor functions.

Results

In order to determine the identity of the second regulator of *dps* expression, a variety of methods were used. First a transposon mutagenesis strategy coupled with a screen for *dps* expression was attempted but due to a poor conjugation frequency in the $\Delta oxyR$ mutant the efficiency of this strategy was too low to pursue. As an alternative strategy, the tBOOH sensitivity assay was used to screen mutated strains lacking oxidative stress regulatory genes. We reasoned that in the absence of the second regulator we would see a change in phenotype. The results of these experiments are detailed in this chapter.

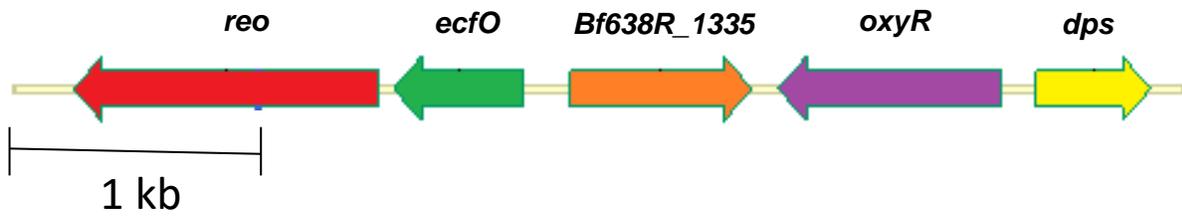
EcfO/Reo are not involved in the tBOOH resistance response. *B. fragilis* possesses many ECF sigma factors which in many other organisms, are known to respond to a variety of stimuli and induce changes in gene transcription. This type of

sigma factor typically works in conjunction with an anti-sigma factor which represses the activity of the sigma factor until an appropriate stimulus is detected at which point the sigma factor is free to induce transcription of its regulon. It has been shown in *B. fragilis* that after aerobic exposure a large number of transcription factors including 14 ECF sigma factors are induced (28, 71). This includes the ECF sigma factor EcfO which works in conjunction with its anti-sigma factor Reo. Further work has demonstrated that the $\Delta ecfO$ mutant had an increased sensitivity to extended oxygen exposure and various oxidative stress inducing agents (71). A regulon for EcfO was determined to contain seven genes. The genes within this operon are all of unknown function but do contain several members of the novel NigD superfamily found only in the *Bacteroidetes* and several members that are lipoproteins (71, 72). It is still unclear as to what role EcfO is playing in the POST response.

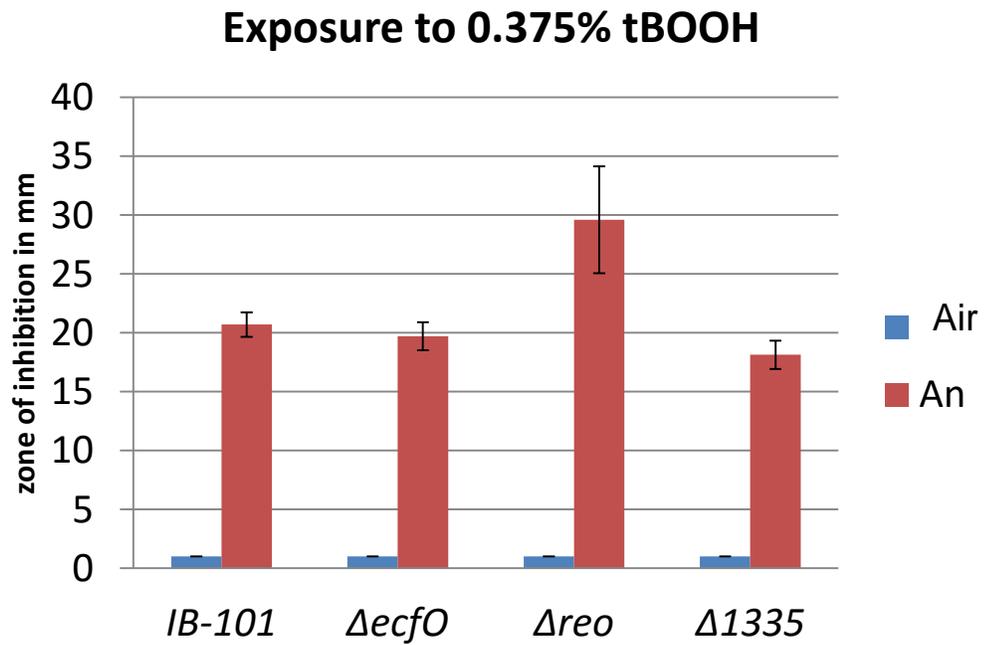
Interestingly *ecfO*, *reo*, and a gene in the regulon, Bf638R_1335 (Bf_1335), are located in the genome adjacent to *oxyR* and *dps* as depicted in Fig. 3.1 A. We reasoned that perhaps EcfO might be the second regulator of *dps* expression given the close proximity to the *dps* gene. To test this, we performed disk diffusion assays with the $\Delta ecfO$ and the Δreo mutants for sensitivity to tBOOH after aerobic exposure. Given the nature of the function of EcfO and Reo we would expect that if they were involved in *dps* expression that the $\Delta ecfO$ mutant would have a zone of inhibition after aerobic incubation whereas the Δreo mutant may have increased resistance as similar phenotypes have been noted (71). As shown in Fig. 3.1 B, when the $\Delta ecfO$ mutant was exposed to tBOOH it had

Fig. 3.1 Role of EcfO and Reo in *dps* expression. A. A schematic representation of the genetic organization of *ecfO*, *reo*, *dps*, and *oxyR*. Genes are drawn to scale. As can be seen these genes are grouped next to each other in *B. fragilis* chromosome. B. Disk diffusion assays performed with 0.375% tBOOH under anaerobic (red bars) and exposure to air (blue bars). Assays were performed in triplicate over two independent experiments with standard deviations shown.

A.



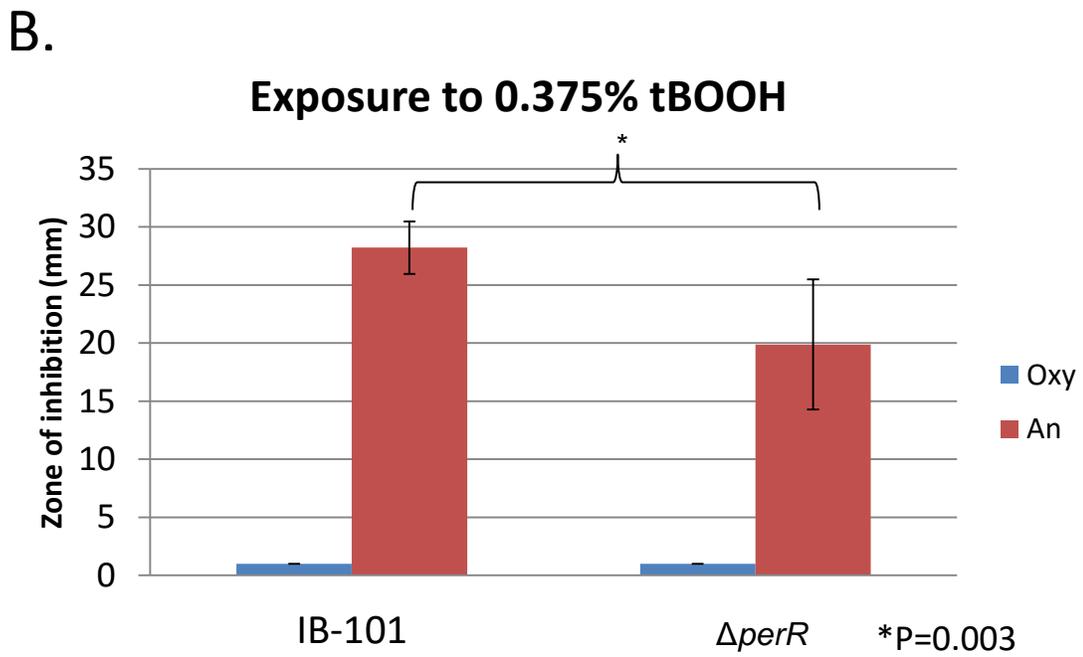
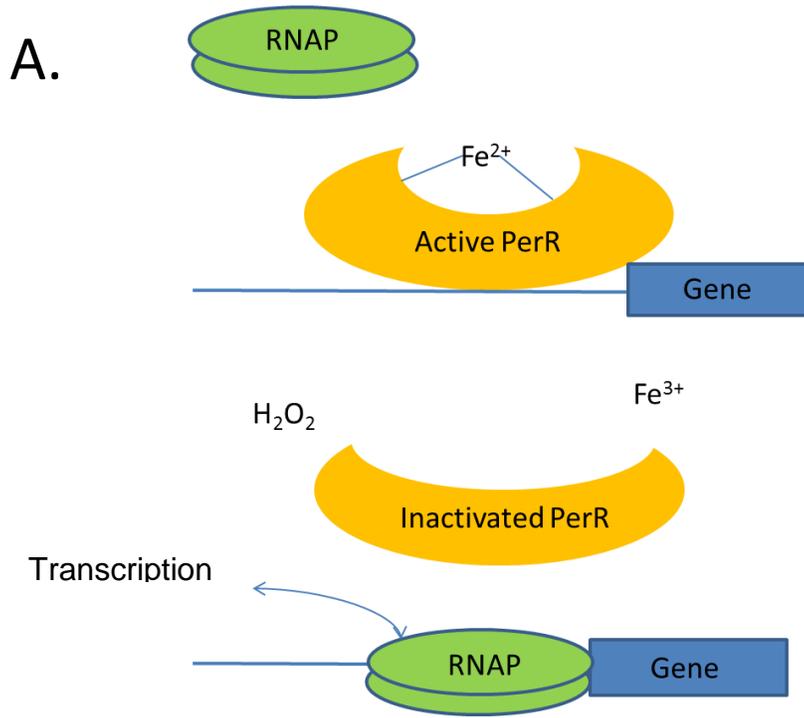
B.



similar zones of inhibition to the WT indicating that EcfO is not involved in this resistance response. The same was seen for a mutant from the EcfO regulon $\Delta 1335$. Interestingly the anti-sigma factor mutant, Δreo , showed increased sensitivity to tBOOH under anaerobic conditions but complete resistance after oxygen exposure. Together this indicates that EcfO is not the secondary regulator of *dps* responsible for resistance seen to tBOOH in the POST response.

PerR affects the tBOOH resistance phenotype but does not affect *dps* expression. One class of regulators that commonly regulate oxidative stress and iron acquisition are those of the Fur (ferric uptake regulator) family of metallo-regulators (109). Members of this family work as repressors and respond specifically to the presence of metals within the cytoplasm and control expression of genes involved in regulating the availability of these metals and limiting their toxic effects. PerR (peroxide resistance regulator) is a member of this family and in *Bacillus subtilis* is the major oxidative stress regulator in this organism (108). In *B. subtilis*, PerR binds iron when it is plentiful which leads to an active repressor that then prevents the transcription of several genes such as *ahpCF*, *katA*, and *mrgA* a *dps* homologue (108). As shown in Fig. 3.2A, when cells experience oxidative stress H_2O_2 accumulates and reacts with the metal containing site of PerR resulting in the release of the iron molecule and a loss in transcriptional repression occurs.

Fig. 3.2 The activity of PerR influences the tBOOH resistance response. A graphic representation of the regulation mechanism of PerR. When cells are not experiencing oxidative stress, iron is bound to PerR and the repressor function is active. However, in the presence of increased levels of H₂O₂, the iron is released from PerR and the repressor becomes inactive allowing for transcription. B. Disk diffusion assays of the $\Delta perR$ mutant were performed with 0.375% tBOOH under anaerobic (red bars) and after three hours of aerobic exposure (blue bars). Assays were performed in triplicate over two independent experiments. Standard deviation is shown and a student's T-test was performed P-value is indicated by the *.



B. fragilis contains three Fur homologues *furA*, *furB*, and *furC*. As previously mentioned *furB* was given the *perR* designation and will be referred to as *perR*. Because of the role that PerR plays in resistance to oxidative stress in a wide variety of other organisms we wanted to investigate *B. fragilis* PerR and determine what role it plays in resistance to tBOOH. To do this we performed disk diffusion assays as shown in Fig. 3.2 B. Interestingly the $\Delta perR$ mutant had increased resistance to tBOOH under anaerobic conditions which is similar to $\Delta perR$ mutants in other organisms which demonstrated increased resistance to oxidative stress (81, 108). These data were consistent with the possibility that PerR was the regulator of *dps* expression. It was determined that further investigation of the role in *B. fragilis* was warranted and that the PerR regulon should be determined.

To elucidate the PerR regulon, we performed microarray analysis to determine the change in gene transcription patterns of the whole genome. To do this cultures were grown to an OD_{550} of 0.5 and then split. Half of the culture was immediately harvested and the remaining culture was exposed to aerobic shaking for three hours. RNA was purified from cells, converted to double stranded cDNA, and sent for microarray analysis. The PerR regulon was determined by comparing gene transcription patterns under anaerobic conditions and genes that have a greater than 5 fold increase in transcription in the $\Delta perR$ mutant compared to WT were identified. These results can be seen in Tables 3.3 A and B.

Table 3.3 Gene expression patterns in the $\Delta perR$ mutant. A. Shows the difference in transcription patterns of *dps*, *bfr*, and *ftnA* under anaerobic conditions. B. Genes that are part of the PerR regulon based on microarray analysis. Column 3 is the fold increase in transcription in the $\Delta perR$ mutant as compared to WT.

A.

| Gene_Tag | Function | $\Delta_{perR/WT}$ |
|-------------|------------------|--------------------|
| BF638R_1333 | Dps | 1.225 up |
| BF638R_3305 | DpsL/ <i>bfr</i> | 1.727 up |
| BF638R_2891 | FtnA | 2.693 down |

B.

| GENE_TAG | FUNCTION | $\Delta_{perR/WT}$ |
|--------------|--|--------------------|
| BF638R0477 | putative exported protein | 41.4 |
| BF638R3069 | putative exported protein | 35.4 |
| BF638R1171.2 | putative type I DNA restriction-modification | 31.6 |
| BF638R2559 | putative lipoprotein | 18.2 |
| BF638R3068 | Putative penicillin-bindin protein | 17.2 |
| BF638R2520 | putative exported transmembrane protein | 10.4 |
| BF638R2532 | tyrosine site-specific recombinase | 9.8 |
| BF638R3888 | putative zinc metaloprotein | 9.5 |
| BF638R3051 | putative membrane protein | 9.2 |
| BF638R4484 | putative lipoprotein | 9.2 |
| BF638R1440 | putative transmembrane protein | 9.0 |
| BF638R0793 | putative polysaccharide biosynthesis protein | 8.9 |
| BF638R0573 | putative aspartate kinase | 8.7 |
| BF638R1630 | hypothetical protein | 8.0 |
| BF638R3066 | putative periplasmic protease | 7.7 |
| BF638R0796 | putative transmembrane polysaccharide modification protein | 7.6 |
| BF638R3253 | putative lipoprotein | 7.1 |
| BF638R1941 | hypothetical protein | 6.7 |
| BF638R3258 | putative membrane protein | 6.6 |
| BF638R3388 | putative lipoprotein | 6.6 |
| BF638R2028 | putative transcriptional regulator MarR family | 6.4 |
| BF638R0771 | conserved hypothetical protein | 6.2 |
| BF638R2661 | putative mobilization protein | 6.1 |
| BF638R3801 | putative RNA polymerase sigma factor | 6.0 |
| BF638R3922 | hypothetical protein | 5.9 |
| BF638R0795 | conserved hypothetical protein | 5.9 |
| BF638R0694 | putative transmembrane immunity protein | 5.9 |
| BF638R4138 | putative outer membrane lipoprotein | 5.8 |
| BF638R4317 | putative autotransporter | 5.8 |
| BF638R3213 | putative exported protein | 5.8 |
| BF638R0408 | putative transporter | 5.8 |
| BF638R2399 | putative lipoprotein | 5.7 |
| BF638R0808 | putative transposase | 5.6 |
| BF638R3722 | conserved hypothetical protein | 5.5 |
| BF638R0787 | putative transmembrane protein | 5.5 |

If a gene was regulated by PerR we would expect to see the transcription significantly increased under anaerobic conditions due to absence of the repressor. Comparing the expression patterns of *dps* under anaerobic conditions in both the WT and the Δ *perR* mutant we did not see a significant increase in transcription as seen in table 3.3 A. These results indicate that PerR is not the second regulator of *dps*. We also looked at the transcription patterns of DpsL (*bfr*) and FtnA . Neither demonstrated an increase in the Δ *perR* mutant. These data indicate that the PerR regulator is not responsible for controlling the transcription of *dps*, *bfr*, or *ftnA*. We also compared the transcription patterns of cultures that were exposed to air and saw similar results.

When comparing the transcription patterns in microarray analysis of the Δ *perR* mutant and WT we were able to determine a putative PerR regulon. As shown in Table 3.3 B those genes that experienced a significant increase in transcription in the Δ *perR* mutant (5 fold or greater) were grouped into this putative PerR regulon. Unfortunately, all the genes that can be grouped into this regulon are genes of unknown function. Among these were genes for putative lipoproteins and putative membrane proteins indicating the PerR regulon may play a role in modifying the membrane during periods of stress. Further work is needed to elucidate the role of PerR tBOOH resistance but it does not appear that PerR is the second regulator of *dps*.

***dps* is not part of the Fur regulon and does not respond to excess iron.**

Many organisms such as *E. coli*, *B. subtilis*, and *Neisseria gonorrhoeae* utilize Fur to control the expression of the iron storage proteins (81). Iron bound in the Fur iron binding cleft does not react with H₂O₂, therefore as long as iron is in excess it will be

bound to Fur and the protein will continue to repress gene transcription. When iron concentrations become limited, Fur will become inactive and gene transcription will occur. Fur works in conjunction with many different regulators and genetic regulatory elements in order to provide sufficient iron for essential activity without allowing for too much which can result in significant damage to the cells (81). We questioned whether the *B. fragilis* Fur could influence the expression of *dps* or *bfr* (DpsL). In an effort to elucidate the role for Fur, microarray analysis was performed with the *B. fragilis* Δfur mutant and WT grown in iron limited conditions and iron replete conditions. Results are shown in Table 3.4.

Table 3.4. Microarray results from analysis of expression patterns of Δfur mutant.

A. Columns 1 and 2 contain the gene number designation in strain BF638R (IB-101) and the putative function based on gene homology. Columns 3, 4, and 5 display the results of the microarray analysis indicating the fold increase measured. Column 3 represents the results when WT in iron depleted conditions (-Fe) was compared to WT under normal growth conditions (+Fe). Column 4 shows results from the Δfur mutant and WT under normal growth conditions. Column 5 shows results from Δfur mutant under iron depleted conditions when compared to WT under normal growth conditions. B. Shows the expression patterns of *dps*, *bfr*, and *ftnA* under the same conditions as detailed above. Columns 3-5 in B correspond to the same conditions as described in A.

A.

| GENE_TAG | FUNCTION | WT -Fe/ WT +Fe | <i>fur</i> +Fe/ WT +Fe | <i>fur</i> -Fe/ WT+Fe |
|------------|---|-------------------|---------------------------|--------------------------|
| BF638R1275 | alkyl hydroperoxide reductase subunit F | 39.2 | 20.4 | 30.4 |
| BF638R1276 | alkyl hydroperoxide reductase C subunit | 36.6 | 12.6 | 25.7 |
| BF638R1422 | hypothetical protein | 8.4 | 17.9 | 17.3 |
| BF638R1421 | putative transmembrane ferrous transport fusion protein (FeoAB) | 5.8 | 13.3 | 9.7 |

B.

| Gene_TAG | Function | WT-Fe/WT +Fe | <i>fur</i> + Fe/WT +Fe | <i>fur</i> -Fe /WT + Fe |
|-------------|------------------|--------------|------------------------|-------------------------|
| BF638R_1333 | Dps | 6.2 | 1.1 | 4.0 |
| BF638R_3305 | DpsL/ <i>bfr</i> | 4.9 | 0.9 | 3.0 |
| BF638R_2891 | FtnA | 1.067 down | 1.279 | 1.005 |

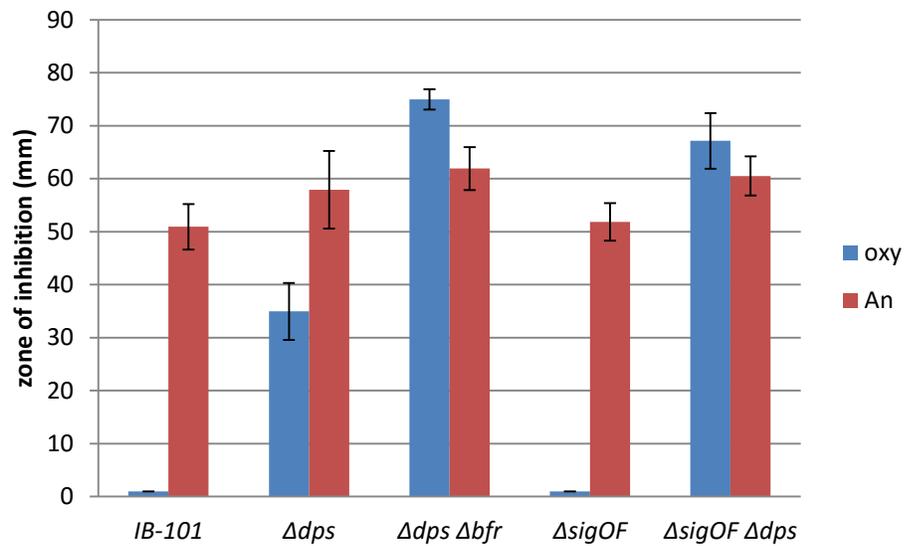
In table 3.4 A the microarray data show that the Fur regulon is limited with few iron responsive genes in the putative regulon. Four genes were found to be both iron responsive and regulated by Fur. Those genes are *ahpC*, *ahpF*, BF638R1421, and *feoAB*. This was determined by looking at transcription levels under all three conditions and observing genes that were iron responsive in WT and were dysregulated in the Δfur mutant. Similar to other organisms, the *B. fragilis* ferrous iron transporter is controlled by Fur to regulate the uptake of ferrous iron. AhpCF is also controlled by Fur which is unusual for true Fur homologues. In fact it is more common for PerR homologues to regulate the transcription of oxidative stress genes as is seen in *B. subtilis* and *Deinococcus radiodurans* (81). What is more interesting is that the Fur regulated expression of *ahpCF* would occur under iron limited conditions which would be less of a concern for oxidative stress given that there is less iron available to participate in the Fenton reaction. There is no known function of BF638R1422 and a homology search indicates that it is specific to the *Bacteroides* only. It is contained in an operon with *feoAB* and therefore could play some role in iron uptake but that has yet to be tested.

Shown in Table 3.4 B are the expression levels of *dps*, *bfr* and *ftnA*. Expression of *ftnA* does not change across all tested condition indicating it is not part of the Fur regulon or iron responsive. The expression levels of *dps* and *bfr* are similar across all tested conditions although there was a slight increase in the expression under iron limited conditions. However when the expression levels of *dps* and *bfr* are compared in the Δfur mutant and WT under normal growth conditions expression levels were similar.

These data indicate that Fur is not the second regulator of *dps* or a regulator of *bfr* in *B. fragilis*.

The role of SigOF as a regulator of *dps* and *bfr* expression. Current work is focused on the role of SigOF in the POST response (Ndamukong, Smith unpublished data). This regulator was identified as one of the 14 ECFs that were regulated by oxygen (28) along with EcfO. Recently SigOF was found to regulate *bfr* induction after prolonged exposure to oxygen thus it was tested in the tBOOH assay. As detailed in Chapter 2, DpsL only shows a phenotype in the Δdps mutant background Fig. 2.3. Therefore when the $\Delta sigOF$ mutant was assayed it was not surprising that it did not demonstrate any defect in resistance to tBOOH Fig. 3.3. We reasoned that because Dps was still present it was able to protect the cells from tBOOH thus the effects of SigOF would be masked in the single $\Delta sigOF$. Thus a double $\Delta sigOF \Delta dps$ mutant was generated to determine whether this mutant was still resistant to tBOOH. The disk diffusion assays showed that the double $\Delta sigOF \Delta dps$ mutant was more sensitive to tBOOH than the Δdps mutant and was similar to the double $\Delta dps \Delta bfr$ mutant. Because the effect of adding the $\Delta sigOF$ mutant to the *dps mutant* was additive this suggested that loss of SigOF caused the increased sensitivity to tBOOH similar to the phenotype in the double $\Delta dps \Delta bfr$ mutant. Taken together these data support that SigOF is the regulator controlling the expression of *bfr* during the POST response.

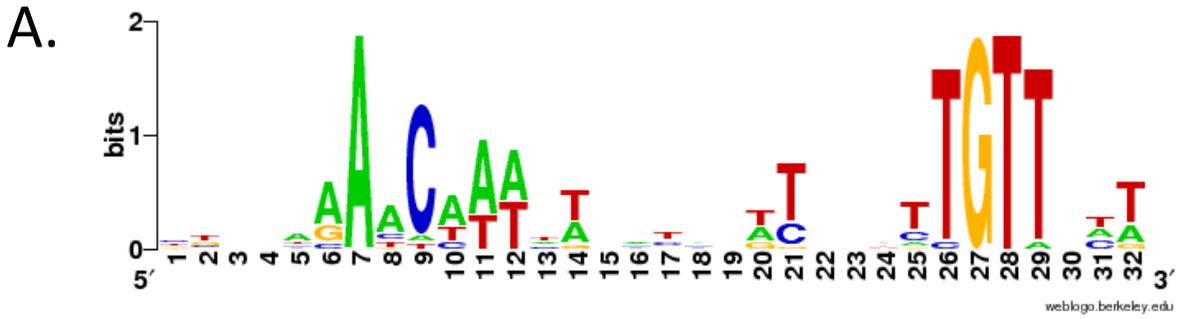
Fig. 3.3 The effect of SigOF on resistance to tBOOH after prolonged exposure to air. Disk diffusion assays were performed as previously described where cells were exposed to 55mM tBOOH. Blue bars represent assays that received three hours of oxygen exposure and red bars represent the results from assays that were kept under anaerobic conditions. Assays were performed in triplicate over two independent experiments. Averages of these are reported with standard deviation shown.



Based on RNA seq and microarray data analysis, a putative SigOF regulon was generated and a promoter recognition sequence was established (Ndamukong, unpublished data). The promoter logo can be seen in Fig. 3.4 A. Interestingly when the *dps* intergenic region was analyzed a SigOF promoter sequence was observed 50 base pairs upstream of the start codon (shown in bold in Fig. 3.4 B) for Dps. This observation suggested that SigOF also was the second regulator of *dps* expression. To evaluate the role of SigOF and OxyR in the POST response tBOOH disk diffusion assays were performed and the results are shown in Fig. 3.4 C.

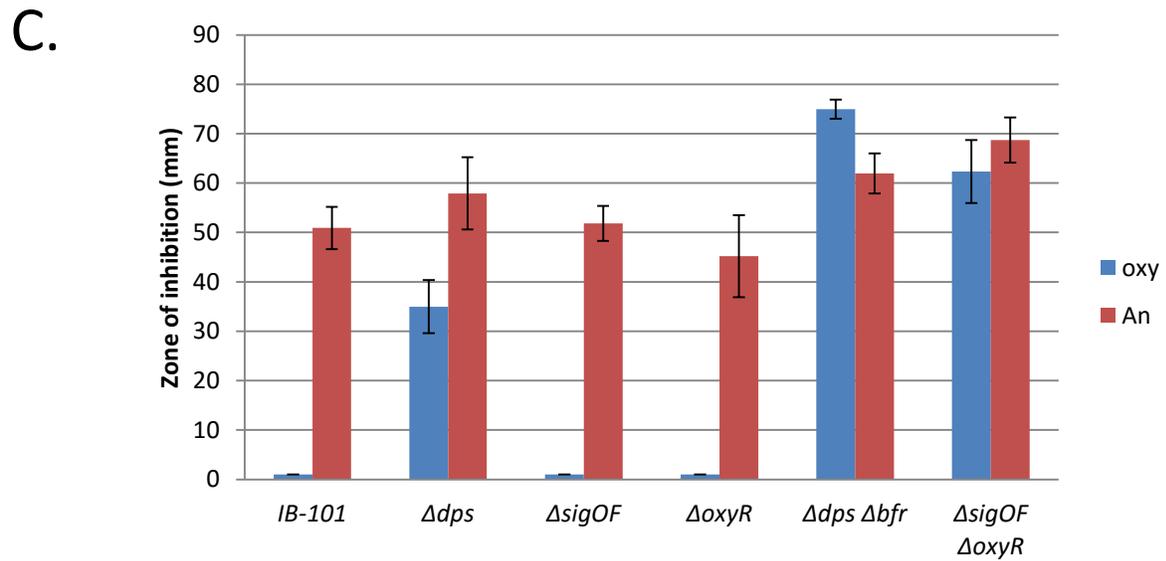
The hypothesis was that a double $\Delta sigOF \Delta oxyR$ mutant would be sensitive to tBOOH after exposure to air as there would be no or limited induction of *dps* expression. As shown in Fig. 3.4 C, the Δdps mutant was the only single mutant that demonstrates sensitivity to tBOOH after oxygen exposure and both of the $\Delta sigOF$ and $\Delta oxyR$ single mutants demonstrated full resistance to tBOOH after aerobic exposure. As previously shown, the double $\Delta dps \Delta bfr$ mutant was more sensitive to tBOOH than the Δdps mutant. Most interesting was the sensitivity of the double $\Delta sigOF \Delta oxyR$ mutant. This mutant showed high sensitivity to tBOOH after aerobic exposure similar to the results seen for the double $\Delta dps \Delta bfr$ mutant. This indicates that the double $\Delta sigOF \Delta oxyR$ mutant has a defect in *dps* expression that is not seen in the single mutants. Overall this suggests that in the absence of both SigOF and OxyR, *dps* expression is either abolished or so low that the double mutant is not resistant to tBOOH. This is strong evidence that SigOF is the second regulator of *dps* expression.

Fig. 3.4 SigOF regulates the expression of *dps* during the POST response. A. SigOF regulon and logo was generated by Ndamukong et. al (unpublished data). B. The intergenic region of *dps* with the conserved SigOF promoter sequence (in bold) is shown 50bp upstream from the Dps start codon. C. Disk diffusion assays of the double $\Delta sigOF \Delta oxyR$ mutant exposed to tBOOH. Disk diffusion assays were performed as previously described with 55mM tBOOH. Assays were performed in triplicate over two independent experiments. Average zones of inhibition are reported with standard deviation of the population shown.



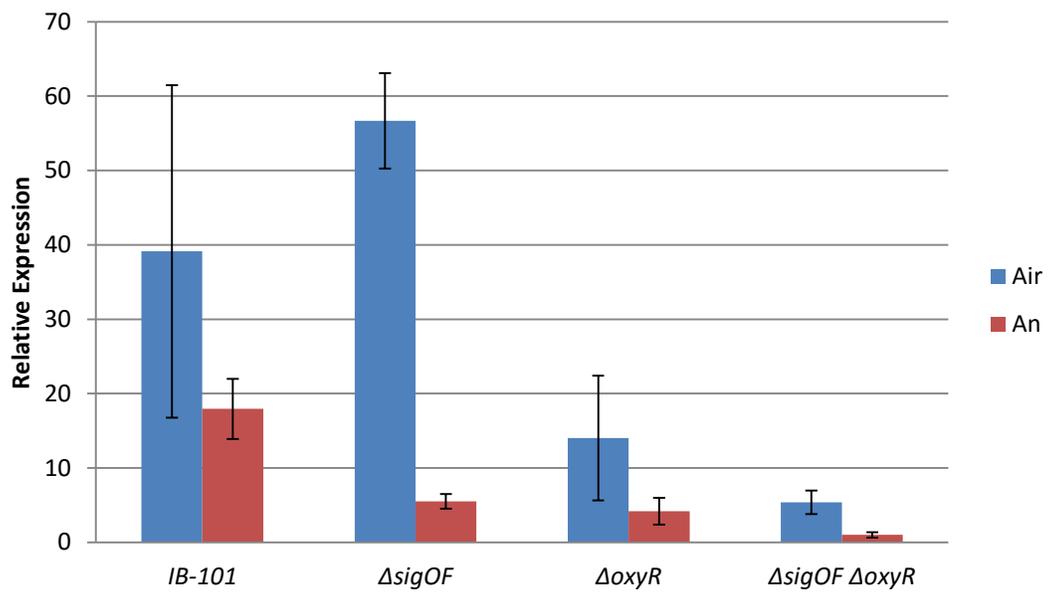
B.

dps intergenic region
 tcatcgttctctgatttattaagatatagaatctatcaatgca
 aagatagaaatcttcagtttgacac**GAATCATT**ttgtgccg
TatctTTGTTccagataaaaagaagaatatagtattaact
 caaaaaaacaatatcattatg



Further evidence for the role of SigOF was obtained from qRTPCR analyses of IB-101, $\Delta sigOF$, $\Delta oxyR$, and the double $\Delta sigOF \Delta oxyR$ mutant cultures shaken aerobically at 37°C for one hour. One hour of aerobic shaking was chosen because previous studies had demonstrated OxyR independent expression of *dps* after 1 hour of aerobic exposure (31). RNA from these cultures were purified, qRTPCR was performed to detect *dps* expression, and results are shown in Fig. 3.5. The expression of *dps* was significantly reduced under anaerobic conditions (red bars). The *dps* expression in the double $\Delta sigOF \Delta oxyR$ mutant was lower than either of the other strains under anaerobic condition and this was statistically significant ($P \leq 0.004$). When the expression of *dps* was measured in the cultures after one hour of aerobic exposure a significant decrease in *dps* was seen when the double $\Delta sigOF \Delta oxyR$ was compared to the WT IB-101 and the $\Delta sigOF$ mutant ($p \leq 0.004$). However when the relative expression levels of *dps* were compared for the $\Delta sigOF \Delta oxyR$ mutant to the $\Delta oxyR$ mutant the decreased expression was not statistically significant. Interestingly the *dps* expression levels measured in each of the three biological replicates in the $\Delta oxyR$ mutant varied greatly as evidenced by the high standard deviation seen in this population. This difference could have resulted from experimental error. Further experiments are needed to elucidate whether the second regulator of *dps* is SigOF but the data presented here supports this idea. Interestingly though, there appears to still be an induction of *dps* expression in the double $\Delta sigOF \Delta oxyR$ mutant of about 5 fold after aerobic exposure. These data could indicate that there may be some other form of induction of *dps* but further experiments are needed in order to determine what is responsible for this induction.

Fig. 3.5 Expression levels of *dps* in the double $\Delta sigOF \Delta oxyR$ mutant are reduced. The average relative expression levels of *dps* for each strain are reported here. Three biological replicates for each strain were measured in duplicate during growth under anaerobic mid-logarithmic phase and post 1 hour aerobic exposure at 37°C. Standard deviation for each population is reported. Relative levels of expression were measured compared to the double $\Delta sigOF \Delta oxyR$ mutant under anaerobic condition as the control.



Discussion

The focus of this chapter was on the identification of the second regulator of *dps* expression in *B. fragilis*. It is not uncommon for multiple regulators to control the expression of *dps* under different conditions. Dps has been shown to promote survival under a variety of conditions including oxidative stress, acid stress, and provides protection from a variety of different stress inducing agents such as hypochlorous acid (50, 51, 81, 111). To respond to these conditions several different regulatory mechanisms are utilized in order to sense the correct conditions and adjust the expression of *dps* appropriately. Regulation of *dps* in *E. coli* is the best studied and several regulators are required for control. These regulators include OxyR, the stationary phase sigma factor σ^S , and two repressors Fis and H-NS (50). In contrast *B. subtilis* utilizes PerR solely to control the expression of the Dps homologue *mrgA* and it uses the stationary phase sigma factor sigma B to control a second Dps homologue (81, 112). As more organisms are studied it has become clear that Dps is utilized in a variety of different conditions and the regulation of this one gene is very important to the overall physiology of the organism.

Dps belongs to the ferritin super family of proteins and it is important to note that organisms that have multiple ferritin super family homologues utilize a diverse series of mechanisms to regulate their expression. There are three stress conditions that modulate expression of these proteins, high iron, oxidative stress, and stationary phase metabolism. *E. coli* utilizes members of the ferritin super family to respond to all three stress conditions listed above. For oxidative stress OxyR induces the expression of *dps*

thus protecting the cell from DNA damage and preventing the Fenton reaction. Additionally the stationary phase sigma factor controls the expression of *dps* to protect the DNA. To manage iron concentrations Fur is used. Under excess iron conditions Fur upregulates the expression of the major iron storage protein FtnA and through a mechanism involving the repression of the small RNA RhyB it induces the expression of bacterioferritin to decrease the levels of iron within the cytoplasm (81).

B. fragilis has three members of the ferritin super family FtnA, DpsL, and Dps. Interestingly all three of these are induced under aerobic conditions (28, 63, 66, 113). Additionally Dps and DpsL are induced during anaerobic stationary phase growth and during the POST response (66, 113). It is interesting to see that *ftnA*, *dps*, and *bfr* (DpsL) are all induced in response to aerobic exposure. This would traditionally be considered a response to oxidative stress but what is interesting is only *dps* is induced strongly in response to H₂O₂ (31). Oxidative stress is considered an accumulation of high levels of H₂O₂ within the cell. In *B. fragilis* the oxidative stress regulator OxyR responds to this excess H₂O₂ and induces the expression of a regulon of genes that are responsible for reducing the levels of H₂O₂ with *dps* being one of the genes induced. These data suggest that Dps is the oxidative stress responsive ferritin superfamily member in *B. fragilis*.

When comparing the induction of ferritins in an anaerobe to that of a facultative aerobe it is important to note one thing. The ferroxidase activity of ferritins and bacterioferritins require oxygen for the conversion of ferrous iron to ferric iron whereas Dps and DpsL utilize H₂O₂. Therefore in the anaerobe *B. fragilis* it would not be

beneficial to induce the transcription of *ftnA* in the absence of oxygen therefore it is logical to have evolved a mechanism that is dependent on the presence of oxygen. FtnA and Bfr have a high capacity for storing iron and in many organisms such as *E. coli* and *N. gonorrhoeae* the iron responsive Fur regulator is utilized either directly in the case of *E. coli* or indirectly in the case of *N. gonorrhoeae* to induce the expression of these ferritins under high iron conditions. Both of these organisms are facultative aerobes and therefore have oxygen present during growth. In addition both organisms have a secondary input that regulates the expression of the iron storage ferritins in response to oxidative stress. In *E. coli*, Fur is directly influenced by H₂O₂ and the activity of OxyR (81). This allows for the input of two stimuli to induce the expression of FtnA and Bfr during high concentrations of iron and oxidative stress. In *N. gonorrhoeae*, Fur senses and responds to high concentrations of iron and represses the iron uptake systems. Fur can also influence the transcription of the small RNA NrrF. When Fur is actively bound to iron it directly represses the transcription of NrrF which then allows for the transcription of *bfr* which reduces the levels of iron within the cell (81).

As shown in Table 3.4, the transcription of *dps*, *bfr* (DpsL), and *ftnA* were similar in both WT and the Δfur . This observation rules out a role for Fur directly regulating the expression of the ferritins in *B. fragilis*. However these experiments were not performed under aerobic conditions and an indirect role for Fur cannot be ruled out. It is possible that Fur is active under conditions of excess iron and it represses a second transcriptional repressor that can promote transcription of (one or more) ferritin genes. It is also possible that a different regulatory network has evolved in *B. fragilis* where both

the presence of oxygen and excess iron are required to induce the transcription of *ftnA*. Further experiments are needed in order to determine what factors contribute to the induction and possible repression of the ferritin genes. An interesting observation from the microarray experiments with the Δfur mutant was that both *dps* and *bfr* (DpsL) were induced under low iron conditions. *B. fragilis* grows slowly under low iron conditions and this may indicate that these cells may be in a transition to stationary phase during which Dps and DpsL might play a role. Further work is needed in order to determine if induction of *dps* and *bfr* occurs in this manner due to stationary phase metabolism.

In many organisms, the PerR repressor regulates the transcription of *dps*. Organisms such as *B. subtilis* and *Streptococcus pyogenes* utilize the dual regulatory abilities of PerR to coordinate the transcription of *dps* (81). PerR directly represses the transcription of *dps* until it is exposed to oxidative stress and increased levels of H_2O_2 . When this occurs the H_2O_2 displaces the iron bound to PerR and the regulator becomes inactive allowing for the transcription of *dps*. Therefore PerR mutants have increased resistance to oxidative stress inducing agents (mainly H_2O_2). This is similar to what was observed for the *B. fragilis* $\Delta perR$ mutant in tBOOH assays under anaerobic conditions Fig. 3.2. However, the PerR regulon suggested by microarray analysis did not include *dps* (Table 3.3). We also did not see an increase in *bfr* (DpsL) expression indicating that the increased resistance to tBOOH was not being conferred through the increased expression of *dps* or *bfr*.

Furthermore the putative PerR regulon was composed primarily of genes that encoded hypothetical proteins and this makes it difficult to deduce what type of stress

this regulon responds to. There are a large number of putative lipoproteins, transmembrane, and transporter proteins so it is possible that this response modifies the permeability of the cells and alters the ability of tBOOH to enter into the cells. Also the genes for two genetic regulators had increased transcription in the $\Delta perR$ mutant, BF638R3801 and BF638R2028. These may influence the transcription of *dps* or *dpsL* but they have never been shown to have increased expression under oxidative stress. Overall these results indicated that PerR is not a second regulator of *dps* and though it influences the tBOOH resistance phenotype it is through an unknown mechanism and does not appear to be regulating expression of the iron storage genes.

The Fur family of proteins are the most common transcription factors used to regulate the acquisition of iron though they do not appear to participate in the control of iron storage in *B. fragilis*. Usually mutations in these regulators will show some form of deregulation of ferritin transcription but this was not observed in the in the $\Delta perR$ and Δfur mutants. This may mean that *B. fragilis* did not evolve a mechanism utilizing the ferritins as iron storage proteins under conditions of high iron. It is possible that *B. fragilis* only needs to respond to high iron concentrations in the presence of oxidative stress and therefore does not require an iron responsive induction of the ferritins. However further work is needed to determine how cellular iron fit into this complicated ferritin regulatory cascade in *B. fragilis*.

As previously shown in Chapter 2, the second regulator of *dps* expression which results in resistance to tBOOH is part of the POST response. The POST response is an extensive genome wide change in transcription patterns that occurs after prolonged

exposure to air (28, 113). Several ECF sigma factors have been shown to become active during the POST response including EcfO, SigOF, and several others (28). Therefore it is possible that these regulators control *dps* expression. As previously mentioned *dps* expression in *E. coli* is controlled by the stationary phase sigma factor σ^S . Though still requiring more investigation, we hypothesize that the POST response and stationary phase have significant overlap. *B. fragilis* does not have a known stationary phase sigma factor however it does have a large number of ECF sigma factors. The POST response is characterized by significant changes in transcription of metabolic genes and a repression of DNA synthesis, translation, and membrane biogenesis which are all characteristic of stationary phase in other organisms (28). Additionally in organisms such as *B. subtilis*, *Staphylococcus aureus*, and *Streptococcus mutans* an ECF sigma factor (RpoE) is utilized to regulate stationary phase (114). Therefore it is reasonable to suggest that the second regulator of *dps* expression may be important in regulation of stationary phase metabolism.

Interestingly, overexpression of *sigOF* resulted in significant upregulation of *bfr* (DpsL) (Ndamukong, unpublished data). A $\Delta sigOF \Delta dps$ mutant was constructed to determine if SigOF was responsible for the POST induction of *bfr* and results showed that the double mutant had similar sensitivity to tBOOH as the double $\Delta dps \Delta bfr$ mutant indicating it was the regulator responsible for *bfr* expression during the POST response Fig. 3.3. Additionally bioinformatic analysis of the *dps* intergenic region revealed a match to the consensus SigOF promoter recognition sequence (Fig. 3.4 A). To investigate the possibility that the second regulator of *dps* is SigOF, a double $\Delta sigOF$

$\Delta oxyR$ mutant was assayed for resistance to tBOOH after aerobic incubation. The double mutant had a high sensitivity to tBOOH after aerobic exposure (Fig. 3.4 B). Interestingly complete resistance to tBOOH after aerobic exposure was seen in both of the single $\Delta sigOF$ and $\Delta oxyR$ mutants. Further studies examined *dps* transcription patterns in the various mutants (Fig. 3.5). Both the single $\Delta sigOF$ and $\Delta oxyR$ mutants expressed *dps* under aerobic conditions. In the double $\Delta sigOF \Delta oxyR$ mutant, *dps* expression was decreased relative to the single mutants providing further evidence that both are controlling the expression of *dps*. This indicates that in the absence of one regulator the second regulator is able to express enough *dps* to protect the cells from tBOOH.

Taken together these data suggest that *dps* expression occurs during both the acute and POST response and is regulated by OxyR and SigOF. This coordinated response promotes the survival of *B. fragilis* under very diverse conditions. Within the intestinal tract, *B. fragilis* has been shown to occupy the intestinal crypts a location that experiences variable levels of oxygen (8, 9). Under normal conditions, the crypts can experience oxygen concentrations up to 8%. However, during periods of nutrient absorption the crypt becomes oxygen depleted. *B. fragilis* may require the coordination of the acute and POST response to survive and thrive in this environment where there are rapid changes in oxygen concentration as well as prolonged exposure to it. It is possible that through this sophisticated oxidative stress response; *B. fragilis* is able to occupy a niche in the crypt which is inhospitable to other organisms. Furthermore, survival within the intestinal tract also requires resistance to the host immune response.

Therefore in the event of an intestinal tear and translocation into the peritoneal cavity, *B. fragilis* is already prepared to survive the increased oxidative stress and the immune responses allowing for survival within the abscess. Through the coordination of the acute and POST response and the coordinated transcription of *dps* and *bfr* (DpsL) *B. fragilis* is able to survive under these diverse and harsh conditions.

CHAPTER 4: SUMMARY

This project details the role of Dps during the acute and POST oxidative stress responses in *B. fragilis*. These studies were the first to demonstrate a role for Dps in the POST response through use of a newly developed phenotypic assay that measures the effect of oxygen exposure on resistance to tBOOH. This assay showed that three hours of aerobic exposure prior to anaerobic incubation was required to induce complete resistance to tBOOH. This extended aerobic incubation is characteristic of the POST response and by assaying several oxidative stress mutants it was demonstrated that the Δdps mutant was sensitive to tBOOH after aerobic exposure. This is the only single mutant that has increased sensitivity in the POST assay. Because of the known role for OxyR in the regulation of *dps* we wanted to determine whether this transcription factor was responsible for the expression during the POST response. Interestingly the $\Delta oxyR$ mutant still demonstrated complete resistance to tBOOH after aerobic exposure indicating that *dps* was expressed during the POST response in an OxyR independent manner. This result indicated that there was a second regulator of *dps* controlled its expression during the POST response.

The similarities between the recently characterized DpsL and Dps led us to consider that DpsL might also be playing a role in resistance to tBOOH. Previous characterization of the DpsL protein indicated that it had ferroxidase activity, a similar structure, and function to the Dps protein. Further work demonstrated expression of *bfr* (DpsL) was during the POST response. Initial tests assayed the Δbfr mutant for resistance to tBOOH but the mutant was completely resistant to tBOOH. We

rationalized that because Dps was still present it was masking the phenotype. Therefore a double $\Delta dps \Delta bfr$ mutant was constructed and it was shown to have a much greater sensitivity to tBOOH than the single Δdps mutant. This indicated that DpsL contributed to tBOOH resistance but to a lesser extent than Dps. DNA damage is the only lethal form of oxidative stress and Dps specifically protects the DNA which may explain why the Δdps mutant is so sensitive to tBOOH. DpsL contributes to resistance to tBOOH indicating a role protecting DNA but it may also have a role in protecting iron containing proteins as well.

Dps and DpsL are known to decrease the concentration of Fe^{2+} in cells therefore the role of iron in tBOOH sensitivity was examined. Cell viability assays were performed in the presence or absence of the intracellular iron chelating agent dipyriddy and then challenged with tBOOH. Cultures treated with dipyriddy were found to be completely resistant to tBOOH whereas untreated cultures were very sensitive. These results indicate that the tBOOH sensitivity is linked to available Fe^{2+} within cells. Therefore it can be inferred that the sensitivity of the Δdps and Δbfr mutants to tBOOH is due to increased levels of Fe^{2+} .

To further study the roles of Dps and DpsL in the survival of *B. fragilis*, *in vivo* survival assays were performed in the rat abscess model. Results demonstrated that the single Δdps and Δbfr mutant did not exhibit an obvious defect in competition with the WT indicating these mutants were not attenuated. Interestingly though, the double $\Delta dps \Delta bfr$ mutant had a significant defect in survival within the abscess indicating that both Dps and DpsL affects the survival of *B. fragilis*. These data indicate that both Dps and

DpsL contribute to survival during infection but the specific role that each are playing and why the absence of both was required to see a defect in survival is still unknown.

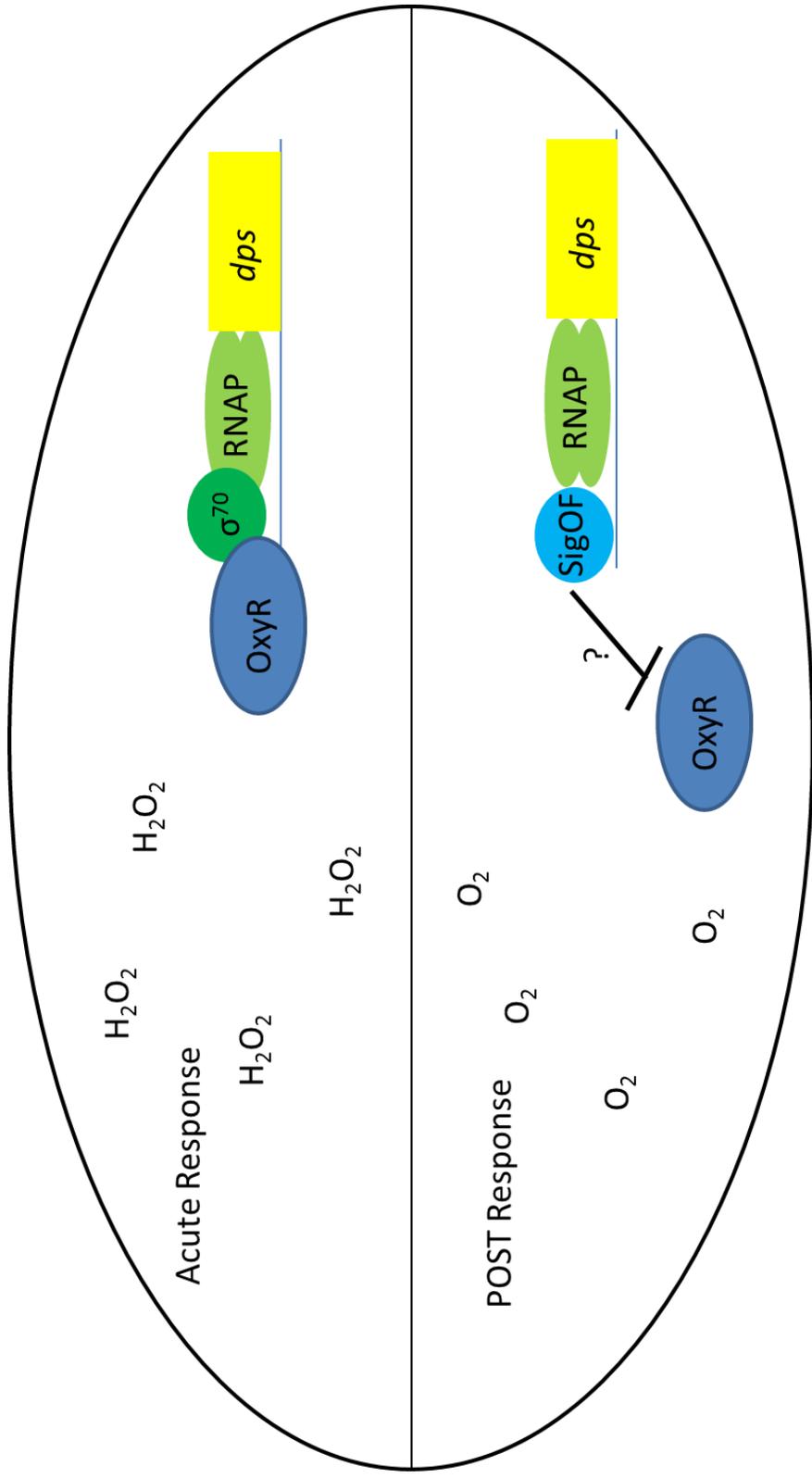
Several strains of *Bacteroides* have Dps, DpsL, and an assortment of ferritins (Ftna1, Ftna2, and Ftna3) but the number and types are not conserved across the genus. Therefore we wanted to determine if this somehow correlated with resistance to tBOOH. The results of these assays showed that the aerobic resistance phenotype was not conserved across the genus and only three *B. fragilis*, *B. ovatus*, and *B. caccae* exhibited an increased resistance to tBOOH after aerobic exposure. This was interesting considering that many other species of *Bacteroides* such as *B. thetaiotaomicron* have Dps and DpsL homologues but not this response. These results indicate that *B. fragilis* and others have developed unique forms of regulation which resulted in the increased resistance to tBOOH.

Understanding the regulation of *dps* during the POST response was dependent on determining the identity of the second regulator. To this end, known oxidative stress regulator mutants were assayed for resistance to tBOOH. Attention was focused on the ECF sigma factor SigOF when bioinformatic analysis demonstrated a consensus promoter sequence for the sigma factor in the *dps* intergenic region. The $\Delta sigOF$ mutant was resistant to tBOOH after aerobic exposure therefore we hypothesized that OxyR control of *dps* expression may provide enough protection for the cells to survive. A double $\Delta sigOF \Delta oxyR$ mutant was constructed and it was found to be highly sensitive to tBOOH after aerobic exposure. This was similar to the double $\Delta dps \Delta bfr$ mutant indicating that SigOF plays a role in the transcription of both *dps* and *bfr* (DpsL) during

the POST response. To further address this possibility we performed qRT-PCR to determine expression levels of *dps* during aerobic incubation in the various mutants. A significant decrease in transcription of *dps* was seen in the double $\Delta sigOF \Delta oxyR$ mutant indicating that these two regulators contribute to the expression of *dps* during exposure to air. However, further work is needed to confirm these results.

Taken together these data demonstrate a multifaceted regulatory network for the control of *dps* and other genes during the acute and POST responses. As shown in Fig. 4.1, the current hypothesis is that *dps* is controlled during the acute response by OxyR which will rapidly induce *dps* leading to a decrease in intracellular ferrous iron and protection of the DNA. As oxidative stress continues and the cells shift to the POST response, *dps* expression is controlled by SigOF and the expression of *dps* can continue throughout this extended stress. In *E. coli* when cells enter into stationary phase, OxyR is prevented from accessing the *dps* promoter. It would be interesting to see if this is also the case in *B. fragilis*. It is possible that the cell is utilizing OxyR to quickly decrease the levels of reactive ferrous iron within the cells and as oxidative stress become prolonged SigOF drives the expression of *dps* but at a lower expression rate to maintain a low level of ferrous iron. It has been shown that OxyR drives a very high level of expression of *dps* upon activation and that the second regulator of *dps* induces a lower expression of *dps* (69).

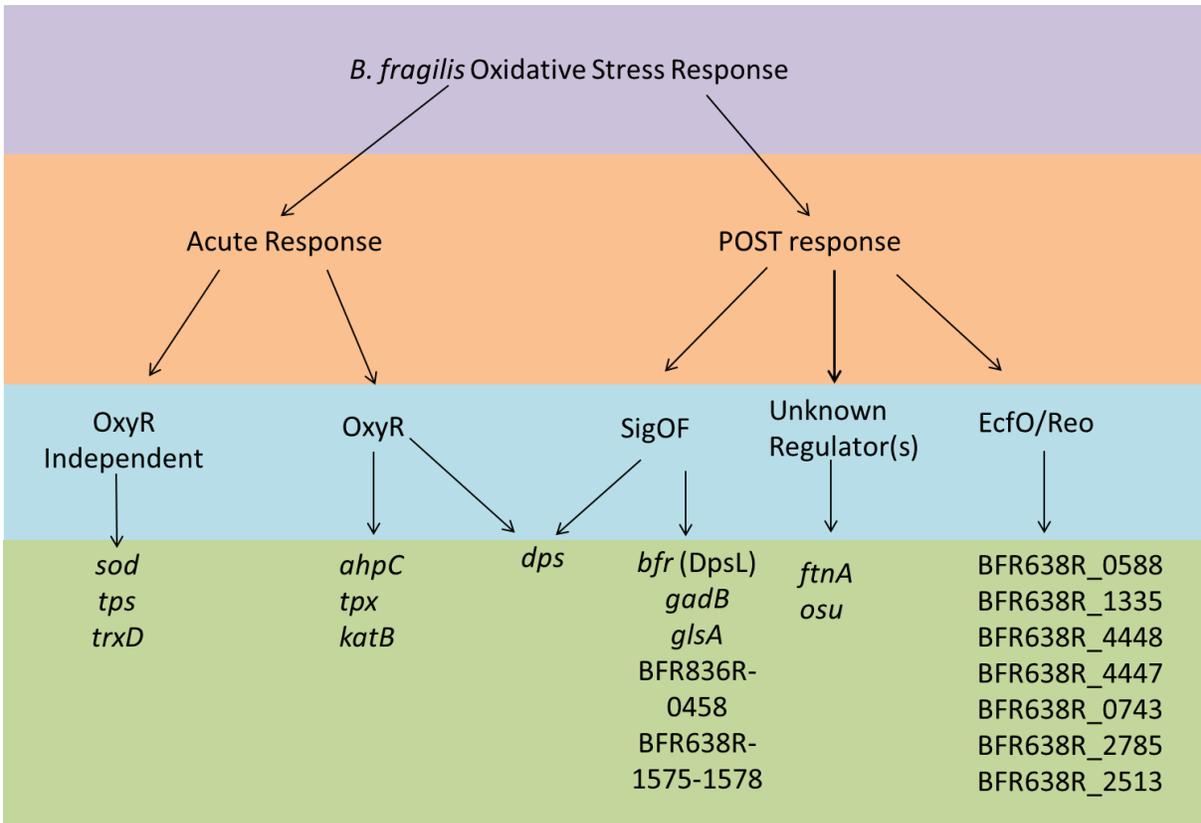
Fig. 4.1 Model of the regulation of *dps* during the Acute and Post response. As shown under the acute response when levels of hydrogen peroxide rise, OxyR becomes activated and recruits σ^{70} to the *dps* gene allowing for transcription. In the POST response SigOF controls the expression of *dps*. Yet to be determined is whether SigOF is able to prevent OxyR from activating the expression of *dps* during the POST response or whether there is some other mechanism that fine tunes the control of the two regulators.



This in turn would keep levels of iron low and would also save cellular resources by producing a lower level of *dps* during periods of extended oxidative stress. It is also possible that SigOF is able to sense and respond to both oxidative stress and iron levels but further investigation is needed. The regulatory cascade network has been updated to represent the dual regulation of *dps* by SigOF and OxyR Fig. 4.2 based on the findings in this report.

Future directions for this project should focus on confirming that SigOF is the regulator responsible for the transcription of *dps* during the POST response. Recently a group working on the closely related *Porphyromonas gingivalis* has shown that a purified ECF sigma factor was able to initiate *in vitro* transcription of the sigma factor specific promoter sequence with purified *E. coli* RNA polymerase (115). A similar approach could be used to show that SigOF can begin *in vitro* transcription of the *dps* gene. The SigOF promoter sequence from the *sigOF* gene or the *bfr* gene could be used as controls in these experiments. Additionally we could make point mutations in the suspected SigOF promoter sequence in the *dps* intragenic region and then see if this mutant was resistant to tBOOH. Ideally this mutation would be made in the Δ *oxyR* mutant and the resulting strain would be sensitive to tBOOH if SigOF was now unable to induce *dps* transcription. That would provide further evidence that SigOF is the second regulator of *dps*.

Fig. 4.2 *B. fragilis* oxidative stress response and *dps* expression. The *B. fragilis* OSR is separated into the acute and post response. The blue represents known and unknown regulators involved in the expression of the genes listed in the green background. Overlap between the two responses can be seen in the regulation of the *dps* gene in the regulation by OxyR and SigOF.



Additionally current data from Ndamukong et al. indicate that SigOF works in conjunction with another ECF sigma factor SigOA. Though the relationship between these two regulators is currently unknown it has been shown that the SigOF regulon is affected by the presence of SigOA. It is possible and very likely that these two regulators have significant overlap in their regulons. It would be interesting to address whether SigOA influences the transcription of *dps*. As seen in Fig. 3.5, expression of *dps* was still induced, though at a reduced level, in the double $\Delta sigOF \Delta oxyR$ mutant after aerobic exposure. It would be interesting to address whether this induction results from the activity of SigOA. To address this an $\Delta oxyR$ mutation could be introduced into the double $\Delta sigOF \Delta sigOA$ mutant to see if *dps* levels are affected and whether there is increased sensitivity to tBOOH

The *dps* gene is commonly induced during stationary phase growth. What has not been addressed is whether SigOF is the stationary phase regulator of *dps*. It is known that *dps* is expressed during the POST response but less is known about its expression during anaerobic stationary phase growth. Expression of *dps* could be determined during stationary phase by qRTPCR. If *dps* is expressed it could be tested whether SigOF induces this expression by observing *dps* expression in the $\Delta sigOF$ mutant during stationary phase. *B. fragilis* has no known stationary phase sigma factor and we currently hypothesize that during stationary phase there are several ECF sigma factors and other regulators that control stationary phase gene expression. It would be interesting if *dps* which has a known role in stationary phase metabolism could be used to study the *B. fragilis* stationary phase (50).

In conclusion the work detailed in this report demonstrates a role for Dps during the acute and POST oxidative stress responses. Future studies using the information provided here could help with the elucidation of the POST response, the roles of SigOF in the physiology and survival of cells, and possibly better define the regulation of stationary phase in *B. fragilis*. These studies have laid the ground work for the further elucidation of the POST response and have shown the connections between Dps and the closely related DpsL. By studying the genetic and post transcriptional regulation of the ferritin family genes and proteins a better understanding of the *B. fragilis* cellular physiology and how this organism uses these proteins for survival will be obtained. This in turn would allow for an increased understanding of the different mechanisms this organism utilizes for survival and overall may lead to better treatment options for this organism during infection.

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Fig. 1.3 Permissions



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Author: George H. Gauss, Michael A. Reott, Edson R. Rocha et al.

Publication: Journal of Bacteriology

Publisher: American Society for Microbiology

Date: Jan 1, 2012

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Author: Michael I. Betteken, Edson R. Rocha, C. Jeffrey Smith et al.

Publication: Journal of Bacteriology

Publisher: American Society for Microbiology

Date: Aug 10, 2015

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Appendix B IACUC Protocol Approval Letters



**Animal Care and
Use Committee**

212 Ed Warren Life
Sciences Building

East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

October 9, 2012

C. Jeffrey Smith, Ph.D.
Department of Micro/Immuno
Brody 5E-106
ECU Brody School of Medicine

Dear Dr. Smith:

Your Animal Use Protocol entitled, "Role of B. Fragilis Oxygen Stress Response in Infection" (AUP #K155a) was reviewed by this institution's Animal Care and Use Committee on 10/9/12. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

A handwritten signature in black ink that reads 'S. McRae'.

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure



**Animal Care and
Use Committee**

212 Ed Warren Life
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252-744-2436 office
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August 11, 2015

C. Jeffrey Smith, Ph.D.
Department of Micro/Immuno
Brody 5E-124
ECU Brody School of Medicine

Dear Dr. Smith:

Your Animal Use Protocol entitled, "Identification of Bacteroides Fragilis Virulence Factors for Intra-Abdominal Infections" (AUP #K170) was reviewed by this institution's Animal Care and Use Committee on August 10, 2015. The following action was taken by the Committee:

"Approved as submitted"

Note: IACUC administratively changed the word pig to ping on page 5 and changed 5 to 6 in the chart on page 11.

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

A handwritten signature in black ink that reads 'S. B. McRae'.

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure