Insights into the Mechanism of Oxygen-mediated Growth Arrest of *Bacteroides fragilis*

A Dissertation

submitted by

Brian Michael Meehan

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Molecular Biology and Microbiology

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

February 2011

Advisor: Michael Malamy

Abstract

The goal of this work was to define the factors that inhibit growth of *Bacteroides fragilis* at oxygen levels > 0.05%. One important aspect of this growth arrest is the accumulation of endogenously-generated reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2). We show here that H_2O_2 scavenging rates were reduced to 20% of the wild-type in a strain missing alkylhydroperoxide reductase (*ahpC*), catalase, and thioredoxin-dependent peroxidase (*tpx*). This strain was hypersensitive to room air and was found to generate ROS at a rate of ~35nM/min/OD_{600}=0.1 under these conditions. However, deletion of fumarate reductase gave rise to a strain that accumulated ~19nM H_2O_2/min//OD_{600}=0.1, indicating that this enzyme accounts for ~47% of the ROS generated by aerated *B. fragilis*. Deleting *frdC* increased the aerotolerance of a Δ*sod* strain by ~100-fold during 9 hours of exposure to room air.

Spontaneous mutants of *B. fragilis* capable of growth under 1% oxygen arise at a frequency of ~10^{-6}. These strains carried mutations in an *orf* designated *oxe*. Deletion of *oxe* established the O_2-enabled phenotype, and this mutant could be re-sensitized to oxygen by providing a wild-type copy of *oxe in trans*. Microaerobic growth of the Δ*oxe* strain was characterized by several amino acid auxotrophies. Though Oxe is not a major source of ROS under room air, a Δ*oxe* strain was found to scavenge micromolar amounts of H_2O_2 at a rate approximately triple that of the wild-type.

Fecal strains of *B. fragilis* did not grow under ~1% oxygen. Ten clinical strains plated microaerobically with efficiencies greater than 10%, 14 plated with frequencies similar to the fecal strains, and 7 had an intermediate phenotype. The *oxe* locus was sequenced for 7 clinical strains that plated with high efficiencies microaerobically and four were found to encode a wild-type Oxe, while 3 were predicted to contain amino acid substitutions in Oxe.

Oxe may contribute to the utilization of amino sugars by *B. fragilis*, as a Δ*oxe* strain bearing a mutant allele of *nagB* could not grow on N-acetylglucosamine or glucosamine. However, the role of Oxe in the anaerobic physiology of this organism is still under investigation.

Table of Contents

List of Tables

CHAPTER 4. Identification of Genes Involved in Growth of *Bacteroides fragilis* on Amino Sugars

List of Figures

Introduction

CHAPTER 2. Characterization of *Bacteroides fragilis* Mutants That Grow Under Increased Oxygen

Introduction

Around two-and-a-half billion years ago, in primordial seas beneath methane clouds, a cyanobacterium acquired the ability to strip electrons from water. In doing so, it ensured unlimited access to an abundant source of the electricity needed to fuel carbon fixation. However, one of the waste products from this photolysis would thoroughly contaminate the atmosphere, thus triggering one of the most dramatic environmental shifts in earth's history. Its appearance would oxidize the ocean's soluble iron, dissipate the methane cloud cover and usher in the first major ice age, and destroy almost every living thing present for the great oxygen catastrophe. But to fully understand oxygen's evolutionary impact, one must appreciate the metabolic strategies established in a pre-aerated world.

A large component of central metabolism in any organism can be described as a complex electrical circuit, in which organic wires transfer electrons to and from various substrates. These so-called "redox reactions" result in reduced and oxidized products, in which the oxidized substance gives up an electron while the reduced product accepts it. Catabolism of carbon sources is a good example of this circuitry in action, as the degradation of large organic molecules releases usable electrons to various cellular acceptors whilst providing the organism with the smaller building blocks necessary for future macromolecular synthesis. These now-reduced electron acceptors can in turn pass their electrical currency onto other substrates or molecules. Eventually the electrical current harnessed through these processes can be converted to stores of chemical energy,

or they can be used to accomplish any number of other important functions, like difficult chemical modifications of crucial molecules or detoxification of harmful substances. In essence, the cell's redox machinery allows for the generation and regulation of an electrical current, and that current can be used to drive whatever metabolic process the cell chooses to "plug in." Ultimately, however, the ability to direct this current requires flexible molecular wires.

Iron Sulfur Clusters

On a pre-oxygenated Earth, life was limited to ancient microbes distantly related to today's bacteria. For these early microbes, construction of molecular wires was made possible by the abundance of two remarkable elements – iron (Fe) and sulfur (S) (52). As a transition metal, iron can adopt various redox potentials and geometries, making it an ideal substance for donating and accepting electrons (52). Its high affinity for sulfur groups meant that it could associate with cysteine residues of extant proteins, thus making it a powerful cofactor for primordial bacteria. These so-called "iron sulfur clusters" (see Figure 1) not only gave these organisms the ability to regulate electrical currents, but allowed for fine-tuning of enzymatic activities by altering metal-coordination spheres (87). In addition, iron-sulfur clusters have a high affinity for organic molecules, so their incorporation into enzymes allowed for interactions with a wide variety of substrates (52). Binding of these substrates was cluster-facilitated as well, as the geometric flexibility allowed for interaction with bulky molecules or protein rearrangement (52). Stability of the protein-substrate interaction was strengthened by the

ability to delocalize electron density over the iron and sulfur (52). So while the assembly of iron-sulfur clusters on bacterial proteins required dedicated machinery akin to the Isc (iron-sulfur cluster) (89) or Suf systems (126), the powerful functionality of these clusters guaranteed their inclusion in many classes of crucial metabolic enzymes. They became involved in substrate binding in dehydratases and radical SAM enzymes, electron transfer reactions in ferredoxins, regulation of gene expression in FNR and SoxR, disulfide reduction in ferredoxin:thioredoxin reductase, and sulfur donation in biotin synthase (56). In short, ancient organisms simplified the complex business of life through the integration of iron-sulfur clusters, but this strategy left them vulnerable to the oncoming oxygen-mediated devastation.

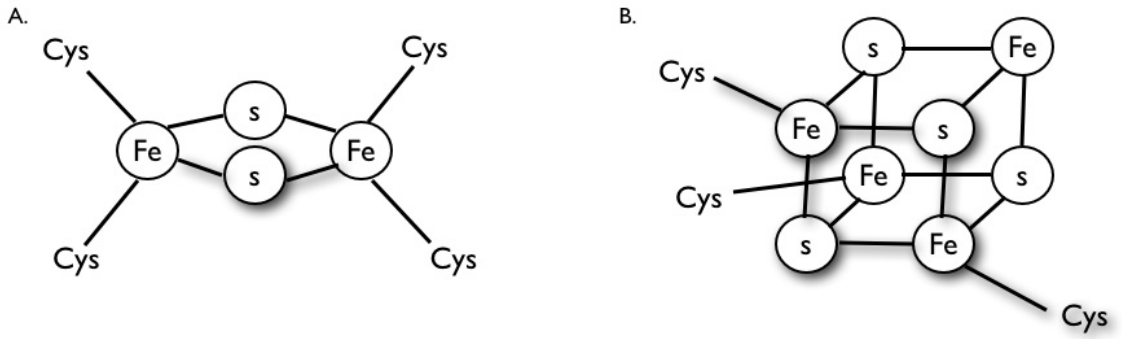


Figure 1. Examples of iron sulfur clusters. A. 2Fe-2S cluster as found in ferredoxin. B. 4Fe-4S cluster as found in aconitase B and pyruvate-ferredoxin oxidoreductase.

The Birth of Oxygen

While the metabolic schemes of primordial bacteria were redox-based, very few electron donors were readily available. Most organisms are thought to have used hydrogen, hydrogen sulfide, or methane (27) to fulfill this role. Sulfates and carbon dioxide acted as electron acceptors, but the energy gained from enzymatic delivery of

electricity to these molecules was moderate (27). Any organism that could circumvent the limitations of these compounds, therefore, would gain a distinct advantage over its energy-poor brethren.

One potential electron donor present in abundance on primordial Earth was water. However, the large energy investment necessary for liberation of electrons from water precluded its utilization. Cyanobacteria were able to overcome this energetic hurdle by exploiting another plentiful resource – high energy solar radiation (27). Thus, photosynthesis was established as an important and powerful metabolic strategy. However, one byproduct of photosynthesis was molecular oxygen (O_2), a gas heretofore unknown to Earth's early inhabitants. While the accumulation of oxygen in Earth's atmosphere did not occur instantly, a slow but steady rise lead to O_2 levels that may have reached the nanaerobic to microaerobic range (<0.08% - 0.3%) during the Archaeon (2.5 billion years ago) (129) (see Figure 2).

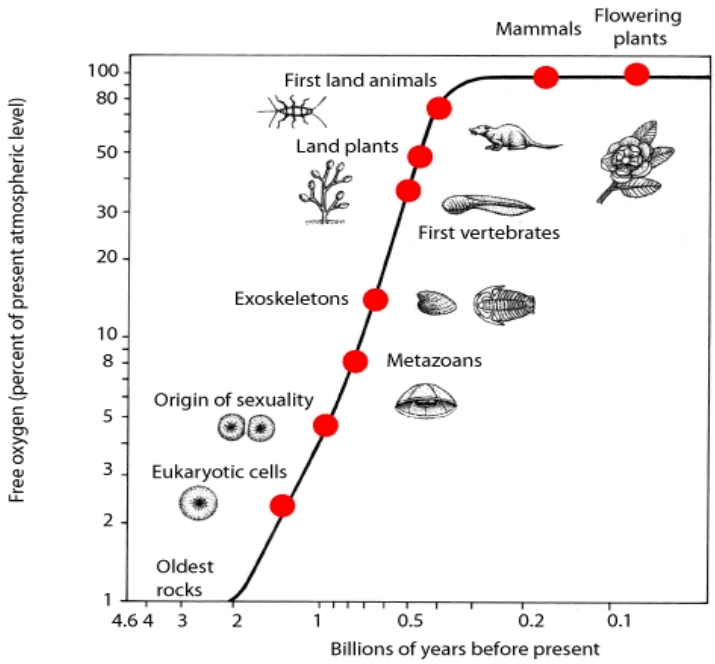


Figure 2. Graph depicting the rise of the earth's atmospheric oxygen levels over time (from (12)), with circles indicating important environmental or biological events.

In one sense, oxygen's introduction was a windfall for these energy-starved cells, as it served as a robust and easily accessible electron acceptor. To take advantage of this feature, many bacteria evolved the ability to reduce this oxygen back to water, thus completing the water-water cycle (27). This aerobic respiration proved to be far more powerful than the anaerobic fermentation pathways utilized previously, leading to an estimated 16-fold increase in the efficiency with which ATP could be generated (118). But this increase in energy capacity came at a price. Concomitant with the increase in O_2 levels was a decrease in levels of atmospheric methane, likely the result of chemical oxidation. Heat previously trapped by these methane clouds via the "greenhouse effect" was thus dissipated and temperatures dropped dramatically. Oceanic waters cooled, leading to the formation of ice as far south as the tropics (68). The effect of this

atmospheric shift was exacerbated by another devastating consequence of oxygenation. The reduced iron (Fe^{2+}) necessary for construction of iron-sulfur clusters was oxidized to its insoluble ferric (Fe^{3+}) form, thus rendering this pivotal element inaccessible (52). Therefore, while the tactical employment of Fe-S clusters as molecular wires by early bacteria proved to be a great advantage under anaerobic conditions, the appearance of oxygen had begun to undermine this strategy. The direct chemical interaction with partially reduced derivatives of oxygen, though, would be the ultimate exposure of these metabolic nodes as potential Achilles's heels.

How Reactive Oxygen Species Form

Molecular oxygen is something of a rarity in that it exists as a stable diradical (51). Two unpaired electrons in its outer valence shell cause the molecule to be relatively reactive with some chemicals, but the process of pairing these electrons is complicated by the fact that they are in the same spin state. For oxygen to pair these electrons in one step, the incoming electrons would have to be of the same spin state relative to one another and opposite to the spin state of the electrons oxygen already possesses. Because no molecules contain spin-aligned electrons in the same orbital, pairing must occur by adding electrons sequentially. However, very few molecules have been described that can efficiently transfer electrons in such a univalent manner (31, 32) . Chief among these molecules are transition metals such as iron (40) and reduced flavins (78), among others.

Addition of electrons to oxygen results in partially reduced derivatives known as reactive oxygen species (ROS). Should O_2 overcome its spin restriction to pair one of its electrons, it forms superoxide (O_2^-), a more reactive chemical than its parent. Pairing the second outer valence shell electron results in the formation of hydrogen peroxide (H_2O_2), and should this molecule proceed to pick up another electron, the end product is the extremely reactive hydroxyl radical (HO^*) (31). Each of these ROS can interact with different classes of biomolecules, and more often than not these interactions lead to destruction or inactivation of the target.

ROS damage

If O_2 could effectively strip electrons from a wide range of substrates, life in an aerobic environment would be impossible (52). The narrow range of potential reactants dictated by oxygen's spin restriction ensures that metabolism can proceed even in the face of oxidative stress. However, molecular oxygen itself can damage enzymes like pyruvate ferredoxin oxidoreductase (PFOR) and nitrogenase (51), thereby inactivating key steps in metabolism. Superoxide has been shown to specifically degrade the Fe-S clusters of several bacterial dehydratases, including dihydroxyacid dehydratase (IlvD), aconitase B, fumarases A and B, and 6-phosphogluconate dehydratase (31, 34). This damage occurs when superoxide oxidizes the cluster, rendering it unstable and leading to its loss from the enzyme (52). H_2O_2 oxidizes cysteinyl residues, which can lead to inappropriate disulfide bonding (51), and like superoxide, hydrogen peroxide can oxidize dehydratases. Even at submicromolar concentrations, hydrogen peroxide can interact

directly with isopropylmalate isomerase (a key component of the leucine biosynthetic pathway) and 6-phosphogluconate dehydratase (55) to inactivate these proteins. Perhaps the most devastating effects of ROS, however, can be traced to their role in the Fenton reaction.

First described in the late 19th century, the Fenton reaction proceeds as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^- \quad (29)$$

This pathway represents the major route of hydroxyl radical generation in the cell, and the iron needed to fuel the reaction is often provided by superoxide-mediated dismantling of iron from Fe-S clusters (10, 60, 61). Following its oxidation by hydrogen peroxide, ferrous iron can be regenerated by NAD(P)H (21, 112) as well as thiols like reduced glutathione (113). *In vivo*, however, these molecules donate electrons at a very slow rate. While accumulation of NADH does, in fact, sensitize non-respiring cells to Fenton-mediated damage, it does so by first reducing free flavins, which recycle ferric iron much more efficiently (133). The crucial role of reduced flavins in iron reduction is exhibited only in non-respiring cells however. Further work revealed that in normally respiring cells intracellular pools of cysteine play the major role in iron reduction (97). The positive charge of Fe^{2+} enables it to interact intimately with negatively charged DNA. Therefore, the hydroxyl radical often forms by interaction of H_2O_2 with this DNA-associated iron, thus potentially leading to DNA lesions in which hydrogen is abstracted from deoxyribose carbon or a hydroxyl group is added to the electron rich double bonds

in the DNA base (40). While the genotoxic effects of this process can be mitigated somewhat by a base excision DNA repair system (47, 53) there is no known mechanism by which to detoxify the hydroxyl radical once formed due to its high rate of reactivity. For the cell to reliably protect vulnerable targets, an impossibly high concentration of scavenger would be needed to intercept the hydroxyl radicals. For this reason, *E. coli* mutants that accumulate submicromolar concentrations of H_2O_2 filament and suffer DNA damage, resulting in a significant increase in mutation rates (97). These effects are the direct result of Fenton chemistry, as addition of the iron chelator desferrioxamine protects these cells.

The exquisite vulnerability of modern-day pathogenic microbes to ROS is exploited by the host immune system, as neutrophils, eosinophils, and mononuclear phagocytes generate superoxide via an NADPH oxidase system associated with the membrane (3, 4). Additionally, Fenton-mediated formation of hydroxyl radical has been implicated in the bacteriocidal effects of gyrase inhibitors (23), hydroxyurea (19) and quinolone, β-lactam, and aminoglycoside antibiotics (64). It is therefore apparent that life in an oxygenated environment requires microbes to constantly defend themselves against an onslaught of oxidative damage.

Anaerobe vs. aerobe

For an ancient bacterium to survive in an oxygen-poisoned world, there appeared to be two potential strategies. It could retreat to the O_2-limited environment of reducing

sediments (26), or it could evolve and adapt to a rapidly changing environment. Those microbes that sought refuge in the sediments are thought to have given rise to modern anaerobes, while the lineage of the second group can be traced to today's aerobes. These aerobic organisms have developed multiple layers of protection against oxidative stress, including superoxide and hydrogen peroxide scavenging enzymes, iron sequestering proteins, and DNA repair systems, to name just a few of the myriad of weapons in their arsenals. By utilizing these tools, aerobes not only tolerated oxygen, but thrived in its presence. It stood to reason, therefore, that anaerobes must not encode many of these oxygen-responsive enzymes, thus relegating them to the few niches free from oxygen penetration. Early surveys indicated an absence of superoxide and peroxide detoxification systems from many oxygen-sensitive organisms (80), but extensive work in the intervening years has conclusively shown the presence of oxidative stress response pathways in many "strict anaerobes." So the factors defining a microbe as an anaerobe or an aerobe remain elusive.

Several provocative theories have been put forth to explain the inability of anaerobes to proliferate in the presence of oxygen. One model posits that overall redox potential may play a significant role, as key metabolic enzymes might have to be in a reduced form to be active (25). Or perhaps oxygen-mediated death occurs in two phases – first electrons used to drive metabolism are diverted toward reduction of oxygen, and second, ROS destroy enzymes and inflict DNA damage (25). While it appears that many anaerobes have functional ROS scavenging systems, it is possible that such systems are outpaced by ROS production in these cells. These theories are by no means mutually

exclusive, and each anaerobic organism may face unique obstacles in the face of oxygen. Work with a number of these bacteria has possibly succeeded only in further muddying the waters of understanding. For instance, *Clostridium acetobutylicum* loses 4 orders of magnitude in viability after 1 hour of aerobic incubation (49) but can maintain low viability for long periods of time, even without forming spores (111). *Desulfovibrio vulgaris*, a sulfate-reducer thought to require anaerobic conditions, actually displayed aerotactic behavior, swimming toward concentrations of O_2 between 0.02-0.04% (0.24-0.48µM dissolved) in capillary tubes in which an oxygen gradient was established (57). Several members of the *Desulfovibrio* genus have evolved an O_2-resistant PFOR in which a disulfide bond triggered by aeration generates a less active, yet more stable enzyme (131). Furthermore, the anaerobes *Moorella thermoacetica, Bacteroides fragilis*, and *Desulfovibrio gigas* have been shown to encode cytochrome bd oxidases, a key component of an oxygen-dependent electron transport system designed to generate ATP (7, 18, 70).

Studies of aerobic microbes have also yielded surprising results. *Bacillus subtilis*, long thought to be a strict aerobe, has demonstrated the ability to grow without oxygen by respiring nitrite or nitrate, and through fermentation (90). Mutants of the facultative aerobe *Salmonella typhimurium* have been isolated that grow only under anaerobic conditions or solely in the presence of oxygen; those lacking topoisomerase I activity were found to be strict anaerobes, while *gyrA* mutants were strict aerobes (134). In light of these findings, the authors concluded that topoisomerase relaxes genomic DNA, and this is necessary for expression of aerobic genes, while gyrase is associated with

supercoiling of chromosomal DNA and is necessary for expression of anaerobic genes. However, an alternative interpretation of these results would suggest that the DNA repair and recombination functions of gyrase (15) are critical under anaerobic conditions, as the SOS response is not activated in Salmonella in the absence of oxygen (22).

Questions of oxygen tolerance are not limited to bacteria. Researchers have described various eukaryotes capable of anoxic growth, as in some unicellular protists (122) as well as a recently discovered metazoan (17). Thus, the line between anaerobes and aerobes is indeed a blurry one.

Bacteroides fragilis

Understanding the molecular mechanisms underlying anaerobiosis requires a good model system. In this regard, the Gram-negative commensal bacterium *Bacteroides fragilis* provides ample opportunity for investigation. As a member of the resident microbiota of the mammalian large intestine, *B. fragilis* feeds on complex polysaccharides found in mucin as well as starches, (75) but also possesses a neuraminidase capable of cleaving sialic acids from the complex glycoproteins and oligosaccharides found to decorate the cells lining the intestinal epithelium (38). The organism can then catabolize these energy sources through a regulatable system unique to members of its class (9), thereby ensuring itself a foothold in its host niche. This microenvironment is thought to be anaerobic, presumably due to the metabolism of facultative anaerobes amongst the colonic flora (69). Therefore, like its ancestors buried

in the earth's reducing sediments, *Bacteroides* can avoid oxidative damage by inhabiting a niche hidden from oxygen's reach.

However, the relationship of *B. fragilis* to its host is not a static one, and damage to the intestinal wall can trigger a virulent response from this microbe. This response can result in the formation of mixed-infection abscesses throughout the body (81), primarily due to the large polysaccharide capsule elaborated by this organism (94). Clinical isolates of *B. fragilis* fall into several classes, and some members possess numerous other virulence factors, including lipases, proteases, heparinases, and nucleases, as well as a membrane –associated beta lactamase (81). In addition, certain strains of *B. fragilis* encode 10 hemolysins (101) and a metalloprotease capable of cleaving E-cadherin in the zone adherens of certain polarized cell lines (115). These features enable *B. fragilis* to outnumber all other *Bacteroides* by 4 fold in cases of clinical sepsis (81).

The primary route of *Bacteroides* transmission is understood to proceed vertically from mother to child during birth (46, 72, 88), but a more generalized fecal-oral route cannot be ruled out, as the organism has been found to persist in municipal sewage waters (119) and to possibly spread amongst hospitalized patients (24). Such transitions would inherently expose the organism to oxygen for substantial lengths of time, as would any journey from the anaerobic confines of the large intestine to sites of infection within the host. How an anaerobe protects itself under such circumstances has become the focus of numerous investigations.

One important piece of the puzzle has revealed that early characterization of the organism was somewhat misleading. Long classified as a strict anaerobe, recent work has demonstrated that *Bacteroides fragilis* can, in fact, benefit from nanomolar amounts of dissolved oxygen (7). By systematically reducing O_2 to water via a cytochrome bd oxidase, the organism can generate a proton gradient conducive to ATP production. In addition, this "nanaerobe" encodes numerous stress-responsive components that aid in reducing the toxicity of oxygen during prolonged exposures.

The B. fragilis Response to Oxygen

Though it has been postulated that oxygen's growth-limiting effect on anaerobes might be due to its role in increasing the overall redox environment (25), this is not the case for *Bacteroides fragilis*. Raising the redox level in growth medium with potassium ferricyanide does not inhibit growth of *B. fragilis* if oxygen is excluded, but introduction of oxygen is bacteriostatic (93, 132). This indicates that O_2 has a specific biochemical effect on the metabolism of this bacterium. In fact, aeration has been shown to cause DNA synthesis in this organism to cease 1-2 hours after aeration. However, RNA and protein synthesis can continue for several hours (114), in direct contrast to the anaerobe *Bacteroides thetaiotamicron*, which ceases macromolecular synthesis immediately upon aerobic exposure (37). 2-D gel electrophoresis indicated that 28 new proteins were upregulated in response to oxidative conditions. Addition of chloramphenicol before aeration lead to a large drop in viability under 20% O_2, implicating these oxidative stress response (OSR) proteins as major determinants of the high aerotolerance of *B. fragilis*

compared to other anaerobes (103). Prominent components of this OSR were small acidic proteins thought to encode alternate sigma factors, implying a global transcriptional response to oxidative conditions. The protein profiles revealed significant overlap between the response to oxygen and to hydrogen peroxide (23 proteins upregulated in mid-log phase cells), thereby raising the argument that the aerotolerance of *B. fragilis* might be due specifically to its ability to cope with reactive oxygen species (103).

Cytochrome bd oxidase

For *B. fragilis*, the first line of defense against oxygen toxicity is to lower its local concentration. This is accomplished through a cytochrome bd oxidase that can efficiently reduce nanomolar amounts of O_2 and couple this reduction to energy production (7). Such heme-only quinol oxidases are normally associated with microaerobic respiration because of their high affinity for oxygen (58). However, this also means that they are not efficient under fully aerated conditions, as the *E. coli* cytochrome bd oxidase has an apparent K_M of 20nM for oxygen (~0.0002% atmospheric) (49). While these oxidases function to conserve energy under microaerobic conditions, they can also protect anaerobic processes such as nitrogen fixation from molecular oxygen (49), and therefore provide *Bacteroides* with an important tool.

OxyR

Should cytochrome bd oxidase not provide adequate reduction of oxygen levels, a rapid and comprehensive response must ensue to protect sensitive intracellular targets from ROS-mediated poisoning. OxyR, an oxygen-sensitive transcriptional response regulator, was first described in *Salmonella enterica* serovar Typhimurium. Activation of the tetrameric OxyR was shown to upregulate numerous proteins required for defense against oxidative stress (13). Early studies in *E. coli* demonstrated that activation occurs when a hydrogen peroxide-catalyzed disulfide bridge forms between two of the proteins six cysteine residues, leading to a conformational change (136). While reduced OxyR binds two major DNA grooves separated by a single helical turn, OxyR containing a Cys199-Cys208 disulfide bond binds four major grooves and can interact with RNA polymerase to promote transcription (86). This oxidization of OxyR can occur at an intracellular H_2O_2 concentration as low as 100nM (40), so the protein was regarded as a finely-tuned sensor of intracellular peroxide levels. Subsequent analysis, however, indicated that the overall internal redox status of the cell also plays a role in the activation state of OxyR. Because the protein is directly reduced by components of the thioredoxin and glutathione systems, inactivation of *trxA* (thioredoxin) along with either *gorA* (glutathione reductase) or *gshA* (glutathione synthetase) gave rise to a constitutively-active OxyR (2).

Several other cysteine modifications of OxyR can take place under stressed conditions, including hydroxylation (OxyR-SOH), nitrosylation (OxyR-SNO), and

glutathionylation (OxyR-SSG). Each of these modifications affects the cooperativity of DNA binding at the third and fourth major grooves, and therefore can lead to differential activation of transcription (62). Such subtlety is indicative of a very sophisticated response to various stresses.

Microarray analysis and 2-D protein profiles indicated that expression of 45% of *B. fragilis* genes are significantly affected by oxygen (103, 124). Among the repressed clusters of orthologous genes (COGs) were gene products responsible for translation and biosynthesis, seemingly priming the organism for entry into a period of dormancy (124). However, COGs associated with energy production, carbohydrate metabolism, sigma factors, cell defense, and nucleic acid repair were all upregulated, indicating that *Bacteroides* was capable of mounting a strong and sustained response to O_2 toxicity (124). A homolog of OxyR was found to play a small role in this response by increasing expression of various enzymes involved in protection from H_2O_2, including various peroxidases, iron-sequestering proteins, and catalase (104, 124) (see Figure 3).

Gene[a]	Symbol	Fold induction[b] Air Wild type	Air *oxyR*	H_2O_2 Wild type	H_2O_2 *oxyR*	5% Wild type	Function and COG Class[c]
Detoxification							
bf1195	*katB*	46	5	28	–	53	Catalase (P)
bf1209	*ahpF*	32	–	70	–	86	Alkyl hydroperoxide reductase subunit (O)
bf1210	*ahpC*	76	–	77	–	93	Alkyl hydroperoxide reductase subunit (O)
bf2360	*tpx*	12	8	7	–	10	Thioredoxin peroxidase (O)
bf2556	*sod*	8	8	–	3	4	Superoxide dismutase (P)
bf2777	*tps*	6	6	–	–	–	Thiol peroxidase-scavengase (O)
bf3656	*glp*	14	16	–	4	–	Glutathione peroxidase (O)
bf2268	*dyp*	5	5	–	–	5	Dyp-type peroxidase (P)
bf2362	*ccp1*	–	–	3	3	–	Cytochrome-C peroxidase (P)
Metal metabolism							
bf1317	*dps*	38	28	25	3	45	DNA and iron binding (P)
bf2884	*ftnA*	4	5	–	–	–	FerritinA, non-haem iron (P)
bf3271	*bfr*	22	18	–	4	–	Bacterioferritin (R)
bf3032	*pir2*	15	16	–	4	6	Pirin (R) iron, cupin family
bf3190	*nlpE*	9	10	–	–	–	Copper resistance; signalling for Cpx pathway (P)
Nucleic acid repair							
bf1191	*nfo*	4	6	–	–	–	Endonuclease IV (L)
bf1780	*radC*	–	–	–	–	3	RecG-like activity (L)
bf1945	*mgs1*	4	6	–	–	–	AAA-family ATPase, Holliday junction repair (L)
bf2642	*uvrB*	–	–	–	–	3	UvrB SOS-repair system (L)
bf3312	*ung*	6	8	–	–	–	Uracil-DNA glycosylase (L)
bf4111	*ada*	3	–	–	–	–	Methylated-DNA – protein-cysteine methyltransferase (L)
Protein repair/chaperones							
bf1172	*clpA*	12	13	–	–	–	Chaperone ATPase (O)
bf3207	*dnaJ*	10	9	–	3	–	DnaJ; chaperon for protein aggregates (O)
bf3378	*msrA*	12	14	–	4	11	Methionine sulphoxide reductase (O)
Redox balance							
bf1279	*trxG*	14	21	–	–	–	Thioredoxin (O)
bf2237	*trxD*	8	12	3	5	13	Thioredoxin (O)
bf2656	*trxX*	5	6	–	–	–	Thioredoxin-like no conserved cysteines (O)
bf2694	*trxC*	7	12	–	4	–	Thioredoxin-exported (O)
bf3015	*trxEF*	7	7	–	4	6	Thioredoxin (O)
General function							
bf2495	*uspA*	16	17	–	3	5	Universal stress protein (T)
bf2778	*oim*	9	10	–	–	–	Membrane protein; HdeD domain, acid defence (S)

Figure 3. List of *B. fragilis* genes that are significantly induced by exposure to 5% oxygen or 50μM H_2O_2. Levels of induction are also shown for an *oxyR* mutant strain (from (124)).

Superoxide Dismutase

In 1969, McCord and Fridovich confirmed the existence of an enzyme capable of detoxifying superoxide through the dismutation reaction outlined below:

$$2\ O_2^- \rightarrow H_2O_2 + O_2 \quad (79)$$

The catalytic efficiency of this superoxide dismutase (Sod) was found to be limited only by the rate of diffusion of its reactants (32). The mere presence of such an enzyme in bacteria underlies the significant damage that superoxide is capable of causing. Indeed, an *E. coli* mutant lacking Sod activity showed a dramatic increase in mutation rates under aerobic conditions (28). This mutant was also auxotrophic for many amino acids, due in part to damage to dihydroxyacid dehydratase (IlvD), and could not utilize non-fermentable carbon sources because fumarase A and aconitase were inactivated (11, 30, 33-35).

Work in *E. coli* has shown that intracellular concentrations of superoxide can be adjusted though differential regulation of *sod* transcription. Using such a system, Gort and colleagues demonstrated that a 2-fold increase in O_2^- lead to inactivation of labile dehydratases, a 4-fold increase lead to growth impairment, and a 5-fold increase lead to oxidative DNA damage. This strongly suggested that *E. coli* makes just enough SOD to allow aerobic growth (41).

Members of the *Bacteroides* genus display large variations in Sod activity (43), though the ability to degrade superoxide is well-documented in *B. fragilis*. A 90-minute exposure to 2% oxygen leads to a 10-fold increase in SodB activity (0.4 to 3.7U/mg) in this organism, and its induction provides significant protection from O_2-mediated death (100). The enzyme is cambialistic, meaning that it can replace its iron co-factor with manganese when oxygen levels rise (44, 100), thus illustrating one example of the link between oxidative stress and iron homeostasis in this organism.

Iron Sequestration

To prevent the onset of Fenton chemistry, *Bacteroides* encodes at least two gene products necessary for protecting soluble iron from interaction with reactive oxygen species. A ferritin homologue has been shown to bind several iron atoms per molecule (102), and its expression is directly regulated by OxyR (109, 124). Also under OxyR control is Dps expression, a dual-action protein capable of binding non-specifically to DNA and coating it while also sequestering and oxidizing Fe^{2+} (1, 124, 135).

Catalase

High concentrations of hydrogen peroxide can be detoxified by catalase via the following reaction:

$$2\ H_2O_2 \rightarrow 2H_2O + O_2$$

B. fragilis encodes a 486 amino acid protein with a surprisingly high degree of similarity to catalases from *Haemophilus influenzae* (71.6% identity to HktE) and to Gram-positive bacteria. The native enzyme is composed of two identical subunits, and is similar to other dimeric catalases in molecular weight (106). It binds heme (42), and is more similar to typical catalases in that it does not have peroxidatic activity (106). Under

anaerobic conditions, the organism displays low catalase activity (0.02U/mg protein), but after 1 hour of aeration, that activity increases substantially (35.6U/mg). Late-stationary cells have increased activity (6U/mg) even under anaerobic conditions (106), indicating that nutritional starvation may in part determine expression levels. This finding was confirmed by the observation that *katB* message was significantly reduced in the presence of glucose under anaerobic conditions (121). Consistent with the enzyme's role in ROS detoxification, *katB* mRNA increases at least 15-fold after addition of 500µM hydrogen peroxide (121), and deletion of the gene sensitizes *Bacteroides* to millimolar concentrations of H_2O_2 (105). Importantly, however, a Δ*katB* strain was not affected in viability when exposed to 20% oxygen (105). While catalase appears to play a role in protecting DNA from oxidative damage, other factors clearly contribute (127).

In investigating the role of ROS detoxification systems in protection of *B. fragilis* from H_2O_2, the Smith lab isolated an intriguing mutant by passaging cultures through increasing concentration of peroxide. This strain maintained viability in up to 50mM H_2O_2, representing a 10-fold increase in tolerance, a phenomenon attributed to increased transcription of the *katB,ahpC*, and *dps* genes (107). Further genetic characterization of this mutant revealed the presence of a missense mutation in OxyR, thus generating a constitutively active variant of this important transcriptional regulator (104). Yet despite the hyperactive peroxide response, this mutant showed *increased* sensitivity to aeration, perhaps demonstrating that aerotolerance is the result of a complex and multifaceted effort, of which ROS detoxification makes up but one part.

Alkylhydroperoxide reductase (AhpC)

Several peroxidases complement catalase for scavenging of hydrogen peroxide. However, unlike the latter enzyme, peroxidases reduce peroxide at the expense of cellular reductants, and do not release oxygen as a product. An example of such a reaction is shown below:

$$H_2O_2 + NAD(P)H + H^+ \rightarrow NAD(P)^+ + 2\ H_2O$$

One important member of this peroxide family is AhpC. So named for its ability to reduce alkyl hydroperoxides, it has been found to most efficiently scavenge H_2O_2, and in fact is the primary detoxification system for endogenously generated hydrogen peroxide in *E. coli* and *Salmonella typhimurium* (99, 117). AhpC receives its reducing power from the flavoprotein AhpF, which in turn accepts electrons from the reduced dinucleotide NADH, although it can also utilize NADPH with a 100-fold less efficiency (98). Kinetic analysis of this peroxidase revealed a K_M of 5μM for H_2O_2, and saturation occurred at 20μM (117), indicating that this system runs most effectively at very low concentrations of substrate. Conversely, the KatG catalase demonstrated a K_M of 5.9mM, suggesting that *E. coli* employs a two-tiered approach to peroxide scavenging. Though catalase can effectively reduce H_2O_2 at high concentrations, the requirement for two molecules of substrate might significantly hinder catalysis as peroxide levels drop. AhpC, on the other hand, requires only one molecule of H_2O_2 for activity, but would quickly exhaust cellular reductants at higher concentrations (117).

The *B. fragilis* enzyme is homologous to this well-characterized AhpC (61% identity and 70% similarity) (108). Transcription of its cognate gene results in a monocistronic message as well as a polycistronic message with the downstream *ahpF*, and a 60-fold increase in *ahpC* mRNA occurs upon exposure to 20% O_2 or H_2O_2, indicating a role analogous to the *E. coli* system (108). While *ahp* mutants were more sensitive to organic peroxides, confirming the role of AhpC in scavenging these reactive species, they did not show increased sensitivity to H_2O_2. These strains gave rise to 5-fold more fusidic acid-resistant mutants than wild-type, but H_2O_2 exposure did not increase this mutagenicity (108). Nevertheless, strains disrupted in *ahpCF* lost viability more quickly than wild-type when aerated (108). Work in the closely related *Porphyromonas gingivalis* also showed significantly upregulated transcription of *ahpC* under 6% oxygen (71), suggesting that these anaerobes may rely heavily on AhpC for protection from endogenous ROS sources.

Tpx and thioredoxins

One of the main differences between various peroxidases is the nature of the immediate reductant. Whereas AhpC receives reducing power from its flavoprotein reductase, other systems tap into electron pools by varying means. For example, while the strict anaerobe *Clostridium acetobutylicum* does not appear to encode AhpC, it demonstrates significant amounts of NAD(P)H-peroxide reductase activity via a thiol-specific antioxidant (TSA) homologue (59). *B. fragilis* encodes a 2-cysteine TSA with an N-terminal Cys in a conserved FVCP motif known to be required for activity. BLAST

analysis shows a high degree of similarity to other thioredoxin-dependent peroxidases (*tpx*) (52% shared identity with *Salmonella enterica* and 48% identical to human), and transcriptional analysis demonstrates that it is part of an operon with the downstream RNA-binding protein (*rbpA*) (48). This operon is partially under the control of OxyR, so exposure to air or H_2O_2 results in an 8.5-fold increase in transcription. Disruption of this operon sensitizes *B. fragilis* to organic peroxides, but not to 3% hydrogen peroxide (48).

In Tpx-mediated peroxide reduction, electrons drawn from NADPH are first transferred to oxidized thioredoxin (Trx) via thioredoxin reductase (TrxB), and then passed from reduced thioredoxin to Tpx. Elucidating the role of this system in the overall ROS response is therefore dependent on an understanding of thioredoxin pools in *Bacteroides*. These small thiol:disulfide oxidoreductases contain the two cysteine redox motif CXXC and are ubiquitous in *B. fragilis* (110). Six *trx* genes dot the genome, and five of them are upregulated by exposure to 20% oxygen (124). Because this bacterium lacks glutathione, thioredoxins may represent its major (or sole) thiol/disulfide redox system, and it is perhaps not surprising, therefore, that a *trxB* mutant is more sensitive to aeration (110).

Other Peroxidases

Four other proteins annotated as peroxidases in *B. fragilis* exhibit upregulated expression under oxidative stress – Glutathione peroxidase (Glp, BF3656), Dye-decolorizing peroxidase (Dyp, BF2268), Cytochrome c peroxidase (Ccp, BF2362), and

Thiol-specific peroxidase (Tps, BF2777) (104, 124). While none have been extensively characterized in this organism, a few interesting details have emerged regarding their functions.

While BLAST analysis of BF3656 indicates homology to various glutathione peroxidases, this classification is most likely erroneous as *Bacteroides* does not encode the glutathione synthase (GshA), and attempts to measure intracellular glutathione have been unsuccessful (110). Therefore, it will be interesting to learn whether this peroxidase draws reductant from thioredoxin pools, or if some heretofore unknown thiol drives reduction of this enzyme.

Little is known about dye decolorizing peroxidases, although structural studies of one DyP revealed a heme cofactor (137).

ccp is located very near to *tpx* on the *B. fragilis* chromosome, and while transcription is induced slightly in the presence of oxygen, hydrogen peroxide elicits a 20-fold increase (124). This protein is typically located in the periplasm, and indeed the *Bacteroides* homologue contains a 19 amino acid signal sequence associated with secretion (48).

Tps is a P20 scavengase homologue, part of a larger group of atypical 2-Cys peroxidases (123). While expression of the *B. fragilis* Tps is induced by oxygen and H_2O_2 exposure, it is not under the control of OxyR. A *tps* mutant was slightly more

susceptible to aeration than wild-type, and the purified protein showed some ability to reduce organic peroxides as well as hydrogen peroxide (123).

The impact of peroxidases on oxygen tolerance would seem to be especially important when comparing *B. fragilis* to other members of the *Bacteroidetes* family. While both *B. thetaiotaomicron* and *Porphyromonas gingivalis* apparently require superoxide dismutase for aerotolerance or recovery from O_2 exposure (91, 96), neither appears to detoxify peroxides in quite the same manner as *B. fragilis*. For instance, while *B. thetaiotaomicron* encodes homologues of AhpC, Glp, Ccp, and Tps, it lacks Tpx and Dyp. *P. gingivalis* has even fewer annotated peroxidases, with genes encoding only AhpC and Tps. However, this organism has been shown to mount a global response to oxidative stress that is partially controlled by OxyR (85, 92), and to utilize a rubrerythrin to reduce both oxygen and H_2O_2 (125). The degree of aerotolerance of these bacteria have not been directly compared, which might shed light on the efficacy of these different ROS detoxification schemes.

Other oxygen-responsive components

Although strong ROS detoxification systems are a must for maintaining viability in the face of ever-present oxygen toxicity, numerous other components make substantial contributions to the cause. Analysis of *B. fragilis* mutants hypersensitive to aeration revealed the presence of a Bacteroides aerotolerance (Bat) operon. The presumed complex encoded by this operon is made up of proteins rich in tetratricopeptide repeats

necessary for protein-protein interactions, and most likely functions in delivering reducing power to the periplasm (128). This may have a generalized role in soaking up periplasmic O_2, or the reductants could be directed to specific sets of redox proteins.

Should protective measures become overwhelmed, repair systems must limit oxidative damage before the organism succumbs to its lethal consequences. To this end, *B. fragilis* encodes a class Ia ribonucleotide reductase (NrdAB), an enzyme mediating the reduction of ribonucloside 5'-diphosphates through a mechanism requiring molecular oxygen (120). This most likely complements the class III ribonucleotide reductase the bacterium uses under anaerobic conditions by maintaining a pool of dNTPs under aeration, thereby facilitating repair of damaged DNA (120). This repair is mediated by homologous recombination, as a *recA* mutant no longer tolerates brief exposures to oxygen (14).

Finally, because a major target of ROS damage is Fe-S cluster containing dehydratases, *Bacteroides* appears to employ a RIC-type (Repair of Iron-sulfur Clusters) protein for repair of clusters inactivated by oxidative or nitrosative stress (BF3316) (95). In this way, central metabolism can continue to run despite the threat of ROS damage.

B. fragilis metabolism

Like all bacteria, the electronic circuitry driving the metabolism of *B. fragilis* relies on electrons derived from the catabolism of carbon sources. Due to a lack of

suitable electron acceptors for respiration, the organism subscribes to a fermentative lifestyle. Liberated electrons are collected by dinucleotides (NAD^+, FAD^+), and shuttled to various enzymes. In addition, the organism utilizes several ferredoxins and flavins, electron carriers capable of performing electron transfer reactions at the very low redox levels found in anaerobically growing cells (10). Many Bacteroides funnel these electrons though an electron-transport chain that allows for ATP synthesis via establishment of a proton gradient (20).

Macromolecular building blocks are generated through a modified TCA cycle, in which a heme-dependent (reductive) and heme-independent (oxidative) branch provide two mechanisms for generation of α-ketoglutarate (6). Crucial to the reductive branch are a fumarase (*fumB)* and a fumarate reductase (*frd)* (67). A *frd* mutant showed a three-fold lower growth yield on glucose compared to WT, indicating that the fumarate reductase pathway plays a key role in generation of ATP, most likely by generating a proton gradient across the inner membrane, which in turn is used to fuel a canonical F-type ATP synthase, similar to the strategy employed by *Wolinella succinogenes* and many other anaerobes (5, 8, 67, 76). Meanwhile, the oxidative branch of the TCA cycle features a mitochondrial-like aconitase (AcnA) necessary for the conversion of citrate to isocitrate (6).

In the closely-related organism *B. thetaiotaomicron*, it has been shown that the major products of fermentation are succinate and acetate when cells are grown on

glucose, indicating that the reductive branch of the TCA cycle is important, as is the PFOR-mediated pathway for conversion of pyruvate to acetyl-CoA (96) (see Figure 4).

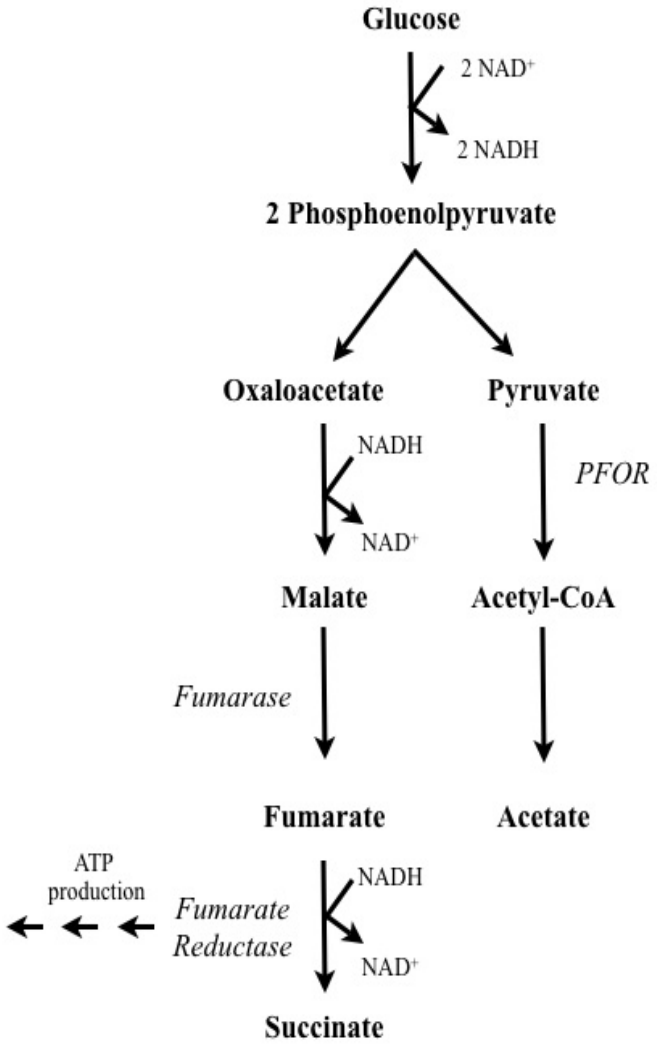


Figure 4. Putative pathway for fermentation of glucose in *B. thetaiotaomicron*, as adapted from (96). Fumarate reductase's role in ATP production is inferred from work in *B. fragilis*.

Pan and colleagues showed that upon aeration, succinate production ceased, and acetate production was lowered (96). Further work revealed that these blocks in

fermentation were due to the sensitivity of fumarase to ROS, and to the sensitivity of PFOR to molecular oxygen. The amount of superoxide dismutase activity in the cells should have been sufficient to protect fumarase if ROS was generated at the same rate as in *E. coli*, leading the authors to conclude that perhaps *B. thetaiotaomicron* generated ROS at a faster rate than its *E. coli* counterpart when shaken in room air (96). While shifting the bacteria to an anaerobic environment restored some of the activity of these enzymes, the authors reasoned that the lability of these important enzymes to oxygen could be a definitive explanation for the inability of *Bacteroides* to grow in room air (96).

Interestingly, *E. coli* encodes several fumarases, including an O_2-resistant FumC (74). In addition, it has at least two aconitases, one of which, AcnA, is active in stationary phase but can be upregulated under oxidative stress conditions (16, 45). This variant form of aconitase is far more stable than the AcnB version, which is the major TCA cycle enzyme in growing cells (130). Therefore, it would appear that some portion of the oxygen sensitivity of an organism might lie in whichever enzymes it utilizes to drive central metabolism.

Endogenous sources of ROS

Clearly, several redundant and complementary systems work together in bacteria to protect crucial targets from reactive oxygen species. Brief exposure to these poisons should therefore prove little threat to microbial life. However, aeration leads to chronic oxidative stress, stemming from partial reduction of O_2 by intracellular electron donors.

Many potential endogenous sources of ROS have been identified through *in vitro* work, and include xanthine oxidase (79), flavohemoglobin (HMP) (82), NAD(P)H:polysulfide oxidoreductase (63), flavin reductase (36), free reduced flavins, lipoyl dehydrogenase, and glutathione reductase (78). Most ROS-generating enzymes fall into the flavoprotein class, meaning that they employ FAD or FMN as cofactors. Amongst these, dehydrogenases like acyl-CoA dehydrogenase and DT-diaphorase produce variable amounts of superoxide (77). Flavoproteins acting as electron transferases, like flavodoxin and ferredoxin-$NADP^+$ reductase generate substantial amounts of O_2^-, while oxidases give rise to hydrogen peroxide (77).

Studies of ROS-generating enzymes *in vivo* are harder to come by. The *E. coli* respiratory chain was thought to be the major source of ROS (39, 83), but that was later found not to be the case (116). Fumarate reductase is a major source of superoxide in anaerobically-grown *E. coli* when assayed in the presence of oxygen *in vitro* (50, 84) but *in vivo* deleting *frd* lowered ROS production only when cells were shifted from anaerobiosis to 20% oxygen (66). The major ROS generator in the periplasm of *E. coli* is the electron-carrier menaquinone (65), but cytoplasmic sources in aerobically growing cells have been elusive.

In *Giardia lamblia*, a protozoan susceptible to oxygenation, an NAD(P)H:menadione oxidoreductase (DT-diaphorase) appears to be a major source of ROS (73). Overexpression of this flavoprotein increases O_2 susceptibility, and leads to an

accumulation of more H_2O_2, while a strain in which DT-diaphorase levels were knocked down demonstrated decreased O_2 susceptibility (73).

Superoxide forms at a rate of approximately 5μM/second in *E. coli* strains missing catalases and peroxidases, and H_2O_2 forms at about 15μM/s (51, 54). These rates were determined by measuring the rate of H_2O_2 accumulation in the extracellular milieu and normalizing to total intracellular volume (numbers of cells x intracellular volume per cell). These production rates vastly exceed the bacterium's metabolic threshold, as superoxide-mediated inactivation of several dehydratases can be significant at concentrations as low as 0.1nM (54). Identification and characterization of endogenous sources of ROS therefore remain important goals in understanding the nature of oxygen sensitivity.

Why can't B. fragilis grow in room air?

In this work, we attempt to shed light on several of the molecular mechanisms limiting *B. fragilis* to environments containing very little oxygen. To this end, we have outlined roles for several important ROS detoxification enzymes and uncovered a major ROS generator. Additionally, characterization of a mutant strain capable of growth under microaerobic conditions has provided us an opportunity to analyze one way anaerobes might adapt to oxidative conditions.

References

1. Almiron M., a J. Link, D. Furlong, and R. Kolter. 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes & Development 6:2646-2654.

2. Aslund F., M. Zheng, J. Beckwith, and G. Storz. 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. Proceedings of the National Academy of Sciences of the United States of America 96:6161-5.

3. Babior B. M., J. D. Lambeth, and W. Nauseef. 2002. The neutrophil NADPH oxidase. Archives of Biochemistry and Biophysics 397:342-4.

4. Babior B. M., R. S. Kipnes, and J. T. Cumvu. 1973. Biological Defense Mechanisms 52:741-744.

5. Baughn A. D., and Malamy M. H. 2003. The essential role of fumarate reductase in haem-dependent growth stimulation of *Bacteroides fragilis*. Microbiology 149:1551-1558.

6. Baughn A. D., and M. H. Malamy. 2002. A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle. Proceedings of the National Academy of Sciences of the United States of America 99:4662-7.

7. Baughn A. D., and M. H. Malamy. 2004. The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. Nature 427:162-165.

8. Biel S., J. Simon, R. Gross, T. Ruiz, M. Ruitenberg, and A. Kroger. 2003. Reconstitution of coupled fumarate respiration in liposomes by incorporating the electron transport enzymes isolated from *Wolinella succinogenes*. European Journal of Biochemistry 269:1974-1983.

9. Brigham C., R. Caughlan, R. Gallegos, M. B. Dallas, V. G. Godoy, and M. H. Malamy. 2009. Sialic acid (N-acetyl neuraminic acid) utilization by *Bacteroides fragilis* requires a novel N-acetyl mannosamine epimerase. Journal of Bacteriology 191:3629-38.

10. Brzóska K., S. Meczyńska, and M. Kruszewski. 2006. Iron-sulfur cluster proteins: electron transfer and beyond. Acta Biochimica Polonica 53:685-91.

11. Carlioz, A Touti D. 1986. Isolation of superoxide dismutase. EMBO Journal 5:623-630.

12. Christiansen, E Hamblin W. 2007. explanet.info. Exploring the Planets.

13. Christman M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Oxidative Stress and Some Heat-Shock Proteins in *Salmonella typhimurium*. Physiological Reviews 41:753-762.

14. Cooper a J., a P. Kalinowski, N. B. Shoemaker, and A.A. Salyers. 1997. Construction and characterization of a *Bacteroides thetaiotaomicron recA* mutant: transfer of Bacteroides integrated conjugative elements is RecA independent. Journal of Bacteriology 179:6221-7.

15. Cozzarelli N. 1980. DNA Gyrase and the Supercoiling of DNA. Science 207:953-60.

16. Cunningham L., M. J. Gruer, and J. R. Guest. 1997. Transcriptional regulation of the aconitase genes (acnA and acnB) of *Escherichia coli*. Microbiology 143:3795-805.

17. Danovaro R., A. Dell'Anno, A. Pusceddu, C. Gambi, I. Heiner, and R. M. Kristensen. 2010. The first metazoa living in permanently anoxic conditions. BMC Biology 8:30.

18. Das A., R. Silaghi-dumitrescu, L. G. Ljungdahl, and D. M. Kurtz. 2005. Cytochrome bd Oxidase , Oxidative Stress , and Dioxygen Tolerance of the Strictly Anaerobic Bacterium *Moorella thermoacetica*. Journal of Bacteriology 187:2020-2029.

19. Davies B. W., M. A. Kohanski, L. A. Simmons, J. A. Winkler, J. J. Collins, and G. C. Walker. 2009. Hydroxyurea induces hydroxyl radical-mediated cell death in *Escherichia coli*. Molecular Cell. 36:845-60.

20. Dawson K. a, M. C. Preziosi, and D. R. Caldwell. 1979. Some effects of uncouplers and inhibitors on growth and electron transport in rumen bacteria. Journal of Bacteriology 139:384-92.

21. Djaman O., F. W. Outten, and J. A. Imlay. 2004. Repair of oxidized iron-sulfur clusters in *Escherichia coli*. The Journal of Biological Chemistry 279:44590-9.

22. Droffner M. L., and N. Yamamoto. 1983. Anaerobic cultures of *Salmonella typhimurium* do not exhibit inducible proteolytic function of the *recA* gene and *recBC* function. Journal of Bacteriology 156:962-5.

23. Dwyer D. J., M. A. Kohanski, B. Hayete, and J. J. Collins. 2007. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Molecular Systems Biology 3:91.

24. Elhag K. M., and a Senthilselvan. 1988. A serogrouping scheme for the study of the epidemiology of *Bacteroides fragilis*. Journal of Medical Microbiology 27:199-205.

25. Engelkirk P., J. Duben-Engelkirk, and V. Dowell. 1992. p. 1-11. In Principles and Practice of Clinical Anaerobic Bacteriology. Star Publishing Company, Belmont, CA.

26. Falkowski, PG Isozaki Y. 2008. The Story of O_2. Science 540-542.

27. Falkowski P. 2006. Tracing Oxygen's Imprint on Earth's Metabolic Evolution. Science 311:1724-1725.

28. Farr S. B., R. D'Ari, and D. Touati. 1986. Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. Proceedings of the National Academy of Sciences of the United States of America 83:8268-72.

29. Fenton H. 1893. The oxidation of tartaric acid in presence of iron. J. Chem. Soc. Proc. 9:113.

30. Flint D. H., M. H. Emptage, M. G. Finnegan, W. Fu, and M. K. Johnson. 1993. The role and properties of the iron-sulfur cluster in *Escherichia coli* dihydroxy-acid dehydratase. The Journal of Biological Chemistry 268:14732-42.

31. Fridovich I. 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen. Annals of the New York Academy of Sciences 893:13-8.

32. Fridovich I. 1998. Oxygen toxicity: a radical explanation. The Journal of Experimental Biology 201:1203-9.

33. Gardner, PR Fridovich I. 1992. Inactivation-reactivation of aconitase in *Escherichia coli*. Journal of Biological Chemistry 267:8757-8763.

34. Gardner P. R., and I. Fridovich. 1991. Superoxide sensitivity of the *Escherichia coli* 6-phosphogluconate dehydratase. The Journal of Biological Chemistry 266:1478-83.

35. Gardner P. R., and I. Fridovich. 1991. Superoxide sensitivity of the *Escherichia coli* aconitase. The Journal of Biological Chemistry 266:19328-33.

36. Gaudu P., D. Touati, V. Nivière, and M. Fontecave. 1994. The NAD(P)H:flavin oxidoreductase from *Escherichia coli* as a source of superoxide radicals. The Journal of Biological Chemistry 269:8182-8.

37. Glass T. L., W. M. Holmes, P. B. Hylemon, and E. J. Stellwag. 1979. Synthesis of guanosine tetra- and pentaphosphates by the obligately anaerobic bacterium *Bacteroides thetaiotaomicron* in response to molecular oxygen. Journal of Bacteriology 137:956-62.

38. Godoy V. G., M. M. Dallas, T. a Russo, and M. H. Malamy. 1993. A role for *Bacteroides fragilis* neuraminidase in bacterial growth in two model systems. Infection and Immunity 61:4415-26.

39. Gonzalez-Flecha, B Demple B. 1995. Metabolic Sources of Hydrogen Peroxide in Aerobically Growing *Escherichia coli*. Journal of Biological Chemistry 270:13681-13687.

40. González-Flecha B., and B. Demple. 1997. Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli*. Journal of Bacteriology 179:382-8.

41. Gort A. S., and J. A. Imlay. 1998. Balance between endogenous superoxide stress and antioxidant defenses. Journal of Bacteriology 180:1402-10.

42. Gregory E. M., and D. D. Fanning. 1983. Effect of heme on *Bacteroides distasonis* catalase and aerotolerance. Journal of Bacteriology 156:1012-8.

43. Gregory E. M., W. E. Moore, and L. V. Holdeman. 1978. Superoxide dismutase in anaerobes: survey. Applied and Environmental Microbiology 35:988-91.

44. Gregory M. 1985. Characterization of the O_2 -Induced Manganese-Containing Dismutase from *Bacteroides fragilis*. Archives of Biochemistry and Biophysics 238:83-89.

45. Gruer M. J., and J. R. Guest. 1994. Two genetically-distinct and differentially-regulated aconitases (AcnA and AcnB) in *Escherichia coli*. Microbiology 140:2531-41.

46. Grönlund MM, Lehtonen OP, Eerola E K. P. 1999. Fecal Microflora in Healthy Infants Born by Different Methods of Delivery: Permanent Changes in Intestinal Flora After Cesarean Delivery. J Pediatr Gastroenterol Nutr. 28:19-25.

47. Henle E. S., and S. Linn. 1997. Repair of DNA Damage by Iron / Hydrogen Peroxide. The Journal of Biological Chemistry 272:19095-19098.

48. Herren C. 2003. Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. Gene 316:167-175.

49. Hill S., S. Viollet, a T. Smith, and C. Anthony. 1990. Roles for enteric d-type cytochrome oxidase in N_2 fixation and microaerobiosis. Journal of Bacteriology 172:2071-8.

50. Imlay J. 1995. A Metabolic Enzyme That Rapidly Produces Superoxide, Fumarate Reductase of *Escherichica coli*. Journal of Biological Chemistry 270:19767-19777.

51. Imlay J. A. 2008. Cellular defenses against superoxide and hydrogen peroxide. Annual Review of Biochemistry 77:755-76.

52. Imlay J. A. 2006. Iron-sulphur clusters and the problem with oxygen. Molecular Microbiology 59:1073-1082.

53. Imlay J. A. 2003. Pathways of oxidative damage. Annual Review of Microbiology 57:395-418.

54. Imlay J. A., and I. Fridovich. 1991. Assay of metabolic superoxide production in *Escherichia coli*. The Journal of Biological Chemistry 266:6957-65.

55. Jang S., and J. A. Imlay. 2007. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. The Journal of Biological Chemistry 282:929-37.

56. Johnson D. C., D. R. Dean, A. D. Smith, and M. K. Johnson. 2005. Structure, function, and formation of biological iron-sulfur clusters. Annual Review of Biochemistry 74:247-81.

57. Johnson M. S., I. B. Zhulin, M. E. Gapuzan, and B. L. Taylor. 1997. Oxygen-dependent growth of the obligate anaerobe *Desulfovibrio vulgaris Hildenborough*. Journal of Bacteriology 179:5598-601.

58. Jünemann S. 1997. Cytochrome bd terminal oxidase. Biochimica et Biophysica Acta 1321:107-27.

59. Kawasaki S., Y. Watamura, M. Ono, T. Watanabe, K. Takeda, and Y. Niimura. 2005. Adaptive Responses to Oxygen Stress in Obligatory Anaerobes *Clostridium acetobutylicum* and *Clostridium aminovalericum*. Applied and Environmental Microbiology 71:8442-8450.

60. Keyer K., a S. Gort, and J. A. Imlay. 1995. Superoxide and the production of oxidative DNA damage. Journal of Bacteriology 177:6782-90.

61. Keyer K., and J. A. Imlay. 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proceedings of the National Academy of Sciences of the United States of America 93:13635-40.

62. Kim S. O., K. Merchant, R. Nudelman, W. F. Beyer, T. Keng, J. DeAngelo, A. Hausladen, and J. S. Stamler. 2002. OxyR: a molecular code for redox-related signaling. Cell 109:383-96.

63. Kobori H., M. Ogino, I. Orita, S. Nakamura, T. Imanaka, and T. Fukui. 2010. Characterization of NADH oxidase/NADPH polysulfide oxidoreductase and its unexpected participation in oxygen sensitivity in an anaerobic hyperthermophilic archaeon. Journal of Bacteriology 192:5192-202.

64. Kohanski M. A., D. J. Dwyer, B. Hayete, C. A. Lawrence, and J. J. Collins. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130:797-810.

65. Korshunov S., and J. A. Imlay. 2006. Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. Journal of Bacteriology 188:6326-34.

66. Korshunov S., and J. A. Imlay. 2010. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. Molecular Microbiology 75:1389-401.

67. Kroger, A Geisler, V Lemma, E Theis, F Lenger R. 1992. Bacterial fumarate respiration. Archives of Microbiology 158:311-314.

68. Kump L. R. 2008. The rise of atmospheric oxygen. Nature 451:277-8.

69. Leavitt M. 1970. Oxygen Tension in the Gut. New England Journal of Medicine 282:1039-40.

70. Lemos R. S., C. M. Gomes, M. Santana, J. LeGall, a V. Xavier, and M. Teixeira. 2001. The “strict” anaerobe *Desulfovibrio gigas* contains a membrane-bound oxygen-reducing respiratory chain. FEBS letters 496:40-3.

71. Lewis J. P., D. Iyer, and C. Anaya-Bergman. 2009. Adaptation of *Porphyromonas gingivalis* to microaerophilic conditions involves increased consumption of formate and reduced utilization of lactate. Microbiology 155:3758-74.

72. Ley R. E., F. Bäckhed, P. Turnbaugh, C. a Lozupone, R. D. Knight, and J. I. Gordon. 2005. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America 102:11070-5.

73. Li L., and C. C. Wang. 2006. A likely molecular basis of the susceptibility of *Giardia lamblia* towards oxygen. Molecular Microbiology 59:202-211.

74. Liochev, S Fridovich I. 1993. Modulation of the Fumarases of *Escherichia coli* in Response to Oxidative Stress. Journal of Biochemistry and Biophysics 301:379-384.

75. Macy J. M., and I. Probst. 1979. The biology of gastrointestinal bacteroides. Annual Review of Microbiology 33:561-94.

76. Macy J., I. Probst, and G. Gottschalk. 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5’-triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. Journal of Bacteriology 123:436-42.

77. Massey V. 1994. Activation of molecular oxygen by flavins and flavoproteins. Journal of Biological Chemistry 269:22459-62.

78. Mayh S. G., L. G. Howell, and P. C. Engel. 1969. The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochemical and Biophysical Research Communications 36:891-897.

79. McCord, JM Fridovich I. 1969. Superoxide Dismutase: an enzymatic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry 244:6049-6055.

80. McCord J. M., B. B. Keele, and I. Fridovich. 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. Proceedings of the National Academy of Sciences of the United States of America 68:1024-7.

81. McGowan K., and S. L. Gorbach. 1981. Anaerobes in mixed infections. The Journal of Infectious Diseases 144:181-6.

82. Membrillo-Hernández J., N. Ioannidis, and R. K. Poole. 1996. The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide in vitro and causes oxidative stress in vivo. FEBS Letters 382:141-4.

83. Messner K. R., and J. A. Imlay. 1999. The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. The Journal of Biological Chemistry 274:10119-28.

84. Messner K. R., and J. A. Imlay. 2002. Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. The Journal of Biological Chemistry 277:42563-71.

85. Meuric V., P. Gracieux, Z. Tamanai-Shacoori, J. Perez-Chaparro, and M. Bonnaure-Mallet. 2008. Expression patterns of genes induced by oxidative stress in *Porphyromonas gingivalis*. Oral Microbiology and Immunology 23:308-14.

86. Mongkolsuk S., and J. D. Helmann. 2002. Regulation of inducible peroxide stress responses. Molecular Microbiology 45:9-15.

87. Moura I., S. R. Pauleta, and J. J. G. Moura. 2008. Enzymatic activity mastered by altering metal coordination spheres. Journal of Biological Inorganic Chemistry 13:1185-95.

88. Mändar R M. M. 1996. Transmission of mother's microflora to the newborn at birth. Biol Neonate. 69:30-5.

89. Nakamura M., K. Saeki, and Y. Takahashi. 1999. Hyperproduction of Recombinant Ferredoxins in *Escherichia coli* by Coexpression of the Gene Cluster. Journal of Biochemistry 126:10-18.

90. Nakano M. M., and P. Zuber. 1998. Anaerobic Growth of Strict Aerobe (*Bacillus subtilis*). Annual Review of Microbiology 52:165-90.

91. Nakayama K. 1994. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. Journal of Bacteriology 176:1939-43.

92. Ohara N., Y. Kikuchi, M. Shoji, M. Naito, and K. Nakayama. 2006. Superoxide dismutase-encoding gene of the obligate anaerobe *Porphyromonas gingivalis* is regulated by the redox-sensing transcription activator OxyR. Microbiology 152:955-66.

93. Onderdonk a B., J. Johnston, J. W. Mayhew, and S. L. Gorbach. 1976. Effect of dissolved oxygen and E_h and *Bacteroides fragilis* during continuous culture. Applied and Environmental Microbiology 31:168-72.

94. Onderdonk A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett. 1977. The Capsular Polysaccharide of *Bacteroides fragilis* as a Virulence Factor : Comparison of the Pathogenic Potential of Encapsulated and Unencapsulated Strains. The Journal of Infectious Diseases 136:82-89.

95. Overton T. W., M. C. Justino, Y. Li, J. M. Baptista, A. M. P. Melo, J. a Cole, and L. M. Saraiva. 2008. Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulfur centers. Journal of Bacteriology 190:2004-13.

96. Pan N., and J. A. Imlay. 2001. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. Molecular Microbiology 39:1562-71.

97. Park S., and J. A. Imlay. 2003. High Levels of Intracellular Cysteine Promote Oxidative DNA Damage by Driving the Fenton Reaction. Journal of Bacteriology 185:1942-1950.

98. Parsonage D., P. A. Karplus, and L. B. Poole. 2008. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. Proceedings of the National Academy of Sciences of the United States of America 105:8209-14.

99. Poole L. B., and H. R. Ellis. 1996. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. Biochemistry 35:56-64.

100. Privalle C. T., and E. M. Gregory. 1979. Superoxide dismutase and O2 lethality in *Bacteroides fragilis*. Journal of Bacteriology 138:139-45.

101. Robertson K. P., C. J. Smith, A. M. Gough, and E. Rocha. 2006. Characterization of *Bacteroides fragilis* Hemolysins and Regulation and Synergistic Interactions of HlyA and HlyB. Infection and Immunity 74:2304-2316.

102. Rocha E. 1992. Isolartion of a ferritin from *Bacteroides fragilis*. FEMS Microbiol Lett. 74:207-12.

103. Rocha E. 2003. The complex oxidative stress response of *Bacteroides fragilis*: the role of OxyR in control of gene expression. Anaerobe 9:165-173.

104. Rocha E., G. Owens, and C. J. Smith. 2000. The redox-sensitive transcriptional activator OxyR regulates the peroxide response regulon in the obligate anaerobe *Bacteroides fragilis*. Journal of Bacteriology 182:5059-69.

105. Rocha E., T. Selby, J. P. Coleman, and C. J. Smith. 1996. Oxidative stress response

in an anaerobe, *Bacteroides fragilis*: a role for catalase in protection against hydrogen peroxide. Journal of Bacteriology 178:6895-903.

106. Rocha E., and C. J. Smith. 1995. Biochemical and genetic analyses of a catalase from the anaerobic bacterium *Bacteroides fragilis*. Journal of Bacteriology 177:3111-9.

107. Rocha E., and C. J. Smith. 1998. Characterization of a peroxide-resistant mutant of the anaerobic bacterium *Bacteroides fragilis*. Journal of Bacteriology 180:5906-12.

108. Rocha E., and C. J. Smith. 1999. Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. Journal of Bacteriology 181:5701-10.

109. Rocha E., and C. J. Smith. 2004. Transcriptional regulation of the *Bacteroides fragilis* ferritin gene (ftnA) by redox stress. Microbiology 150:2125-34.

110. Rocha E., A. O. Tzianabos, and C. J. Smith. 2007. Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe *Bacteroides fragilis*. Journal of Bacteriology 189:8015-23.

111. Rolfe R. D., D. J. Hentges, B. J. Campbell, and J. T. Barrett. 1978. Factors related to the oxygen tolerance of anaerobic bacteria. Applied and Environmental Microbiology 36:306-13.

112. Rowley D. a, and B. Halliwell. 1982. Superoxide-dependent formation of hydroxyl radicals from NADH and NADPH in the presence of iron salts. FEBS Letters 142:39-41.

113. Rowley D. A., and B. Halliwell. 1982. Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds. FEBS Letters 138:1-4.

114. Schumann J. P., D. T. Jones, and D. R. Woods. 1983. Effect of oxygen and UV irradiation on nucleic acid and protein syntheses in *Bacteroides fragili*s. Journal of Bacteriology 156:1366-8.

115. Sears C. L. 2001. The toxins of *Bacteroides fragilis*. Toxicon 39:1737-46.

116. Seaver L. C., and J. A. Imlay. 2004. Are respiratory enzymes the primary sources of intracellular hydrogen peroxide. The Journal of Biological Chemistry 279:48742-50.

117. Seaver L. C., J. A. Imlay, and P. Loewen. 2001. Alkyl Hydroperoxide Reductase Is the Primary Scavenger of Endogenous Hydrogen Peroxide in *Escherichia coli*. Journal of Bacteriology 183:7173-7181.

118. Sessions A. L., D. M. Doughty, P. V. Welander, R. E. Summons, and D. K. Newman. 2009. The continuing puzzle of the great oxidation event. Current Biology. 19:R567-74.

119. Shoop D. S., L. L. Myers, and J. B. LeFever. 1990. Enumeration of enterotoxigenic *Bacteroides fragilis* in municipal sewage. Applied and Environmental Microbiology 56:2243-4.

120. Smalley D., E. Rocha, C. J. Smith. 2002. Aerobic-Type ribonucleotide reductase in the anaerobe *Bacteroides fragilis*. Journal of Bacteriology 184:895-903.

121. Smith C. J. 1997. Regulation of *Bacteroides fragilis* katB mRNA by oxidative Stress and carbon limitation. Journal of Bacteriology 179:7033-7039.

122. Stoeck T., F. Bruemmer, and W. Foissner. 2007. Evidence for local ciliate endemism in an alpine anoxic lake. Microbial Ecology 54:478-86.

123. Sund C. J., W. Greg Wells, and C. Jeffrey Smith. 2006. The *Bacteroides fragilis* P20 scavengase homolog is important in the oxidative stress response but is not controlled by OxyR. FEMS Microbiology Letters 261:211-7.

124. Sund C. J., E. Rocha, A. O. Tzianabos, A. O. Tzinabos, W. G. Wells, J. M. Gee, M. a Reott, D. P. O'Rourke, and C. J. Smith. 2008. The *Bacteroides fragilis* transcriptome response to oxygen and H_2O_2: the role of OxyR and its effect on survival and virulence. Molecular Microbiology 67:129-42.

125. Sztukowska M., M. Bugno, J. Potempa, J. Travis, and D. M. Kurtz. 2002. Role of rubrerythrin in the oxidative stress response of *Porphyromonas gingivalis*. Molecular Microbiology 44:479-88.

126. Takahashi Y., and U. Tokumoto. 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. The Journal of Biological Chemistry 277:28380-3.

127. Takeuchi T., Y. Nakaya, N. Kato, K. Watanabe, and K. Morimoto. 1999. Induction of oxidative DNA damage in anaerobes. FEBS Letters 450:178-80.

128. Tang Y. P., M. M. Dallas, and M. H. Malamy. 1999. Characterization of the BatI (Bacteroides aerotolerance) operon in *Bacteroides fragilis*: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in *in vivo* model systems. Molecular Microbiology 32:139-149.

129. Towe K. 2002. The Problematic Rise of Archaen Oxygen. Science 295:1419a.

130. Varghese S., Y. Tang, and J. A. Imlay. 2003. Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. Journal of Bacteriology 185:221-230.

131. Vita N., E. C. Hatchikian, M. Nouailler, A. Dolla, and L. Pieulle. 2008. Disulfide bond-dependent mechanism of protection against oxidative stress in pyruvate-ferredoxin oxidoreductase of anaerobic *Desulfovibrio* bacteria. Biochemistry 47:957-64.

132. Walden W. C., and D. J. Hentges. 1975. Differential effects of oxygen and oxidation-reduction potential on the multiplication of three species of anaerobic intestinal bacteria. Applied Microbiology 30:781-5.

133. Woodmansee A. N., and J. A. Imlay. 2002. Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. The Journal of Biological Chemistry 277:34055-66.

134. Yamamoto N., and M. L. Droffner. 1985. Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe *Salmonella typhimurium*. Proceedings of the National Academy of Sciences of the United States of America 82:2077-81.

135. Zhao G., P. Ceci, A. Ilari, L. Giangiacomo, T. M. Laue, E. Chiancone, and N. D. Chasteen. 2002. Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. Journal of Biological Chemistry 277:27689-96.

136. Zheng M. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science 279:1718-1722.

137. Zubieta C., S. S. Krishna, M. Kapoor, P. Kozbial, D. Mcmullan, H. L. Axelrod, M. D. Miller, P. Abdubek, E. Ambing, T. Astakhova, D. Carlton, H.-ju Chiu, T. Clayton, M. C. Deller, J. Feuerhelm, S. K. Grzechnik, L. Duan, J. Hale, E. Hampton, G. W. Han, L. Jaroszewski, K. K. Jin, H. E. Klock, M. W. Knuth, A. Kumar, D. Marciano, A. T. Morse, E. Nigoghossian, L. Okach, S. Oommachen, R. Reyes, C. L. Rife, P. Schimmel, H. V. D. Bedem, D. Weekes, A. White, Q. Xu, K. O. Hodgson, J. Wooley, A. M. Deacon, A. Godzik, S. A. Lesley, and I. A. Wilson. 2007. Crystal structures of two novel dye-decolorizing peroxidases reveal a b-barrel fold with a conserved heme-binding motif. Proteins 223-233.

CHAPTER 1

Identification of Fumarate Reductase as a Major Source of Reactive Oxygen Species in *Bacteroides fragilis*

Abstract

Despite the catastrophic role that endogenously-generated reactive oxygen species (ROS) may play in bacteria exposed to aerobic environments, very few *in vivo* ROS-producing enzymes have been identified. Studies of these enzymes are often precluded by the presence of efficient and often redundant ROS scavenging pathways. In *Bacteroides fragilis*, catalase (KatB) and alkylhydroperoxide reductase (AhpC) have been shown to prevent accumulation of hydrogen peroxide, yet mutants lacking these gene products are still capable of scavenging H_2O_2 at significantly high rates. In this report, we show that the thioredoxin-dependent peroxidase, Tpx, is responsible for much of the peroxide scavenging found in a Δ*ahpC*Δ*kat* mutant, as a Δ*ahpC*Δ*kat*Δ*tpx* strain eliminated H_2O_2 at approximately 20% of the rate of the wild-type. This triple mutant was not sensitized to 3% hydrogen peroxide in a disk-diffusion assay, but upon exposure to 21% oxygen it lost viability far more quickly than the wild-type. Colony-forming units fell below the level of detection within 60 hours for the triple mutant in this assay, compared to 96 hours for the wild-type, indicating that Tpx plays an important role in detoxifying endogenously-generated ROS.

With a Δ*ahpC*Δ*kat*Δ*tpx* strain we were able to quantify the rate of hydrogen peroxide formation in *B. fragilis* exposed to room air. In such a strain H_2O_2 generated intracellularly can cross the cell membrane and thereby allow for quantification in the extracellular milieu. When anaerobically-grown, mid-log phase cells of this triple mutant were suspended in PBS and glucose to an OD_{600}=0.1 and shaken in 21% oxygen, H_2O_2

accumulated at a rate of 36±6 nM/min in the buffer. In an attempt to identify the enzyme responsible for this ROS production, we made a deletion in fumarate reductase subunit C (*frdC*), as this complex has been shown to contribute significantly to ROS formation in *E. coli* (20). The triple mutant lacking *frdC* gave rise to H_2O_2 at a rate of 19±1 nM/min, indicating that fumarate reductase is responsible for approximately 47% of ROS production in *B. fragilis* under these conditions. We therefore tested whether deletion of *frdC* could increase the aerotolerance of a superoxide dismutase mutant (Δ*sod*) strain shown to be hypersensitive to room air. While approximately 99.9% of Δ*sod* cells were killed by a nine-hour exposure to a fully oxygenated environment, the Δ*sod*Δ*frdC* strain maintained about 14% viability under the same conditions. These results lead us to conclude that Frd is a major ROS-producing enzyme in aerated *B. fragilis* cells.

Introduction

Life in an oxygenated world presents interesting and difficult challenges to organisms from all walks of life. Due to its small size and unpaired outer-orbital electrons, molecular oxygen (O_2) can permeate cells and strip electrons from a wide variety of biomolecules (7, 8, 15). This electrical piracy results in the formation of partially reduced derivatives of O_2 appropriately known as reactive oxygen species (ROS). Such ROS consist of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical, and their effects on the cell include inactivation of integral proteins, damage to DNA, and eventually death (15). To survive in such a climate, an organism must limit the rate of endogenous ROS production, detoxify ROS quickly upon formation, and protect and regenerate key ROS-labile proteins and molecules. To this end, organisms like *E.coli* have evolved many responses to oxidative stress. For example, a shift from an anaerobic environment to one containing oxygen results in the upregulation of ROS detoxifying enzymes like superoxide dismutase (Sod) (23), and peroxidases such as alkylhydroperoxide reductase (AhpC) (13, 27). Because AhpC becomes saturated at H_2O_2 concentrations above 20µM (32), *E. coli* also encodes two catalases that scavenge peroxide at higher concentrations. And should protective mechanisms fail, *E. coli* can produce more-oxidative-stress-resistant versions of critical enzymes like fumarase and aconitase (5, 22, 38) or activate regeneration machinery like the iron-sulfur cluster repair pathway, Isc (6).

Because of its robust response to oxygen, E. coli can easily shift between anaerobic and aerobic environments. Many bacteria, however, do not display such metabolic flexibility and are therefore relegated to niches containing little to no oxygen. These so called "anaerobes" stop growing upon the introduction of O_2, but the nature of this growth cessation is still somewhat mysterious. One interesting and important model for studying the effects of oxygen on anaerobic bacteria is *Bacteroides fragilis*. Long-regarded as a strict anaerobe, this mammalian commensal has been found to benefit from nanomolar concentrations of dissolved oxygen, and yet cannot grow when O_2 levels are increased further (3). However, the bacterium can maintain viability in room air even after several days of exposure, partially due to a Bacteroides aerotolerance (Bat) operon seemingly dedicated to pumping reductant into the periplasm (35). While no *fumC* or Isc-like pathways have yet been identified, a great deal of research has shown that B. fragilis has evolved strong and sophisticated ROS detoxification systems. Superoxide dismutase has been shown to protect the bacterium from O_2 exposure (28) and KatB, the cytoplasmic catalase, has been shown to protect the bacterium from millimolar concentrations of hydrogen peroxide (30). A Δ*ahpC* mutant showed increased sensitivity to O_2 and organic peroxides, but not to 3% H_2O_2 (31). Moreover, deletion of this gene led to a higher frequency of fusidic acid resistant mutants, indicating that AhpC plays an important role in protecting from DNA damaging effects. In addition to AhpC, five other putative peroxidases have been annotated in the B. fragilis genome. Little is known about the genes annotated as a Dyp-type peroxidase (BF2268), glutathione peroxidase (BF3656) or cytochrome c peroxidase (BF2362), but the gene encoding thiol peroxidase (*tps*) (BF2777) has been shown to be upregulated six-fold upon exposure to room air, and its

protein product affords protection against organic peroxides (33). While *tps* transcription is not upregulated in the presence of H_2O_2, the authors conclude that this peroxidase may be important at high H_2O_2 concentrations (33) . A related enzyme, the thioredoxin-dependent peroxidase, is encoded by the gene annotated as *tpx* (BF2360). This gene has been shown to be upregulated by both oxygen and H_2O_2 (12-fold and 7-fold, respectively), and yet a strain with a deletion of this gene again exhibited an increase in sensitivity to organic peroxides, but not to exogenous H_2O_2 (12). These studies indicate that further investigation of the response of *Bacteroides* to ROS is required.

Even less is known, however, about the sources of ROS in *B. fragilis*. As destructive as endogenously-generated reactive oxygen species appear to be, no *in vivo* sources have yet been identified in this organism. With this study, therefore, we sought to uncover major ROS generators while further characterizing the detoxification pathways utilized by this aerotolerant anaerobe.

Materials and Methods

Reagents

Hydrogen peroxide and horseradish peroxidase were purchased from Sigma (St. Louis, MO) and Amplex Red was obtained from Invitrogen (Carlsbad, CA).

Growth conditions

Anaerobiosis was maintained by using a Coy anaerobic chamber containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. *B. fragilis* was grown in brain heart infusion broth supplemented with 0.5% yeast extract and 15µg/ml hematin (BHIS) or anaerobic minimal medium (AMM) containing 0.5% glucose, as previously described (2). In some cases super anaerobic minimal medium (SAMM) plates containing 150µg/ml hemin were used (37). Because all strains are *thyA* mutants, thymine was added to 50µg/ml. Gentamicin (50µg/ml), rifampicin (50µg/ml), trimethoprim (80µg/ml) and tetracycline (2.5µg/ml) were added to plates where appropriate. *E. coli* was grown aerobically in L broth, and chloramphenicol (25µg/ml) and tetracycline (10µg/ml) were added where appropriate.

Strains and Plasmids

Strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α was used for cloning, and strain HB101/pRK231 was used for mobilization of plasmids from DH5α to *B. fragilis* recipient strains. DH5α was made competent for transformation through use of the RbCl method previously described (11).

Strain	Relevant characteristics	Reference
B. fragilis		
ADB77	TM4000 Δ*thyA* Tp^R	(2)
ADB247	ADB77 Δ*frdC247*	(1)
MBD616	TM4000*thyA*$_2$Δ*sod*	lab stock
ADB247/616	ADB247Δ*sod*	
BM50	ADB77Δ*ahpC*Δ*kat*	This work
BM95	ADB77Δ*tpx*	This work
BM105	ADB77Δ*ahpC*Δ*kat*Δ*tpx*	This work
BM112	ADB77Δ*ahpC*Δ*kat*Δ*tpx*Δ*frdC*	This work
E. coli		
DH5a	λ nonlysogen	(39)
HB101	*rpsL20*, host strain for pRK231	(36)
Plasmids		
pRK231	RP4 derivative, Tet^R Tra^+	(9)

pADB242	pYT102 derivative, 0·35 kb *Bam*HI–*Hin*dIII fragment replaced by 18 bp *Bam*HI–*Hin*dIII fragment from pCR2.1-TOPO (Invitrogen) Cm^R	(1)
pADB247	pADB242 derivative with Δ*frdC247* allele	(1)
pADB242Δ*katB*	pADB242 containing deletion construct for *katB*	This work
pADB242Δ*ahpC*	pADB242 containing Δ*ahpC*	This work
pADB242Δ*tpx*	pADB242 containing Δ*tpx*	This work

Table 1. Bacterial strains and plasmids used in this study.

DNA manipulation

Primers used in this study are listed in Table 2. Primer sequences were designed using the *B. fragilis* NCTC9343 annotated sequence found on the Pedant3 webpage (http://pedant.gsf.de) and synthesized by IDT (Iowa City, IA). Genomic DNA was amplified using HotStarTaq Master Mix (Invitrogen, Carlsbad, CA). Plasmid and PCR product purifications were performed with QIAprep spin columns (Qiagen, Chatsworth, CA). Where indicated, DNA was digested with restriction enzymes purchased from New England Biolabs (Beverly, MA). Ligations were performed using T4 DNA Ligase from Invitrogen.

Strain construction

All in-frame deletions of *B. fragilis* genes were created using a two-step double crossover technique (10). In order to build a deletion construct for *ahpC*, a fragment consisting of 683bp of *ahpC* 5' upstream sequence and 94bp of amino-terminal coding sequence was amplified using primers BAM56 and BAM57. A fragment of 48bp of carboxy-terminal *ahpC* coding sequence and 727bp of downstream intergenic and *ahpF* coding sequence was amplified with primers BAM58 and BAM59. After purifying the PCR products, the upstream fragment was digested with BamHI and NcoI restriction enzymes, while the downstream fragment was digested with NotI and NcoI enzymes. These digested fragments were purified and ligated via a three-way reaction with pADB242 that had been digested with BamHI and NotI to create pADB242Δ*ahpC*.

The suicide plasmid pADB242Δ*ahpC* was delivered to the recipient *B. fragilis* strain as previously described (36). Tetracycline-resistant colonies were screened for the appropriate cointegrant event using primers 1843 and BAM59. Isolates demonstrating recombination at the *ahpC* locus were grown overnight in BHIS with thymine to allow for recombination events leading to resolution of the disrupting plasmid. The presence of the *thyA* gene on pADB242 sensitizes cointegrants to trimethoprim, while those resolvants that have excised the plasmid are trimethoprim-resistant. For this reason, the culture was plated to SAMM containing glucose, thymine, and trimpethoprim. Colonies arising after 3-4 days were purified and then screened for tetracycline-sensitivity on

BHIS+thymine plates. Tetracycline-sensitive colonies were then used as template in a PCR with primers BAM59 and BAM60 to identify Δ*ahpC* clones.

The deletion construct for *katB* was created by PCR amplifying a region consisting of 293bp of *katB* 5' upstream sequence and 477bp of amino-terminal coding sequence with primers BAM68 and BAM69 to create the upstream fragment. A fragment of 507bp of *katB* carboxy-terminal coding sequence and 229bp of downstream sequence was amplified with primers BAM70 and BAM71. Again, the upstream fragment was digested with BamHI and NcoI and the downstream fragment was digested with NotI and NcoI. These were ligated to the BamHI/NotI-digested pADB242 to create pADB242Δ*kat*. The procedure described above was used to create cointegrants, which were screened by PCR with primers 61RAB and BAM72. Resolvants were screened with primers BAM68 and BAM71.

pADB242Δ*trx* was created by PCR amplifying a fragment consisting of 763bp of *tpx* upstream sequence, which includes coding sequence for a putative hydrolase as well as intergenic sequence, and 44bp of *tpx* amino-terminal coding sequence to create the upstream fragment. The downstream fragment was made by PCR amplifying a region consisting of 49bp of amino-terminal coding sequence and 761bp of downstream sequence, which includes a ribosome binding protein. These fragments were digested as above, and ligated into pADB242. Cointegrants were screened with primers 61RAB and BAM216 as well as 1843 and BAM219. Resolvants were screened with primers BAM216 and BAM219.

Primer number	Region of homology	Sequence
1843	pADB242	CCCATCGGTGATGTCGGC
61RAB	pADB242	GGCGCGCCGTAAGGAAAGTGGCTCTCAG
BAM56	ahpC1	CGATGGATCCGCAAAGGTAGGGTGAAG
BAM57	ahpC2	TGACCCATGGCCCTTTACGTCTTCGC
BAM58	ahpC3	GGCACCATGGCGAAGCAACCCTGAAAC
BAM59	ahpC4	AGGTGCGGCCGCGATGGAAGTTTCCGCAC
BAM60	ahpC5	CGACTCTTAGGTACTGG
BAM68	kat1	ATATGGATCCATCCCCCTGTGGTA
BAM69	kat2	ATATCCATGGTTAGCGCTACGCATGTT
BAM70	kat3	ATATCCATGGTTGAAGGTATCGGCTTC
BAM71	kat4	ATATGCGGCCGCCTGGGCATTTCTTTG
BAM72	kat5	CTGCACTTTACGCACTG
BAM216	tpx1	ACATGGATCCGCTTCATTAATCTGG
BAM217	tpx2	CAATCCATGGGTGGCATCGAATTTCG
BAM218	tpx3	ACATCCATGGCATGAAAGCTACCGAAG
BAM219	tpx4	CAATGCGGCCGCAAACATCGCTTTAAAG

Table 2. Primers used in this study

H_2O_2 Detection

The protocol for measuring H_2O_2 scavenging was adapted from the method of Seaver and

Imlay (32). Briefly, solutions of horseradish peroxidase (HRP) and Amplex Red (AR) reagent were made in 18ml phosphate-buffered saline (PBS) (pH 7.2) (8g NaCl, 0.2g KCl, 1.44g Na_2PO_4, and 0.24g KH_2PO_4 per liter) to concentrations of 20µg/ml and 56µg/ml, respectively. A H_2O_2 stock of approximately 30µM was made in PBS (pH 7.2) from a 30% solution and the exact concentration was determined by measuring the absorbance of the solution at 240nm in a spectrophotometer (ϵ=43.6M^{-1} cm^{-1}). This solution was further diluted to give a 200µM H_2O_2 stock. Aliquots were added to 500µL HRP mixed with 500µL AR in 4ml polystyrene cuvettes and diluted to a final volume of 2ml with PBS. These samples were briefly shaken by hand and placed in a Aminco Bowman Series 2 luminescence spectrophotomter for reading with an excitation wavelength set to 563 nm and an emission wavelength set to 587nm. Fluorescence readings were recorded and used to construct a standard curve for H_2O_2 concentration. All experiments were conducted in the presence of oxygen.

H_2O_2 Scavenging

Strains to be tested for H_2O_2 scavenging were first grown anaerobically to mid-log phase in BHIS. Cultures were adjusted to an OD_{600}=0.1 in BHIS and placed in foam-stoppered flasks and transferred to a 37°C shaker under room air (21% oxygen). Samples were shaken for 1 hour at 600rpm, at which point they were centrifuged for 10 minutes at 6000rpm. Supernatants were removed, and the cell pellets were washed twice with an equal volume of PBS, as the growth medium was found to significantly reduce the sensitivity of the assay. Following the second wash, cell pellets were resuspended in 10 ml PBS and transferred to Hungate tubes for testing. 250µl of H_2O_2 stock was added to

the 10ml cultures to give a starting concentration of 5µM. Immediately after application of peroxide, the tubes were quickly capped and upended once to mix. 1.1ml was immediately removed to a 1.5ml microcentrifuge tube and spun for approximately 10sec at 6000rpm in a micro centrifuge Model SD110 (Clover Laboratory, Waterville, OH). 1 ml of supernatant was removed to a 4ml polystyrene cuvette (VWR) and 500µL horseradish peroxidase and 500µL Amplex Red solutions were added. The mixture was shaken briefly by hand and immediately placed into the Aminco Bowman luminescence spectrophotometer to be read.

ROS Generation

Strains were grown for 2 days at 37ºC in 5ml AMM. Aliquots of these dense cultures were then used to inoculate 10ml AMM. Cultures were shaken anaerobically at 100rpm in 50ml flasks and grown for several generations. When the OD_{600} reached approximately 0.3, cultures were transferred anaerobically to conical screw-cap tubes, sealed, and centrifuged 5 minutes at 5000rpm. Tubes were brought back into the anaerobic chamber and the supernatants were decanted. Cell pellets were resuspended in 10ml AMM lacking cysteine and transferred to 125ml foam-stoppered flasks. These flasks were shaken at 600rpm in room air at 37°C for one hour. Cultures were again transferred to conical screw-cap tubes and centrifuged as before. Cell pellets were washed twice with PBS and then resuspended in 20mls PBS+0.05% fresh glucose+thymine at an OD_{600} =0.1. Flasks were shaken at 600rpm in room air at 37°C. 1.1ml samples were drawn at various time points as described above to determine H_2O_2 concentrations in the supernatants.

H2O2 Sensitivity

Strains were grown overnight in 5 ml BHIS under anaerobic conditions. 200µL of culture were then spread on BHIS plates that had been reduced in the anaerobic chamber overnight. When the surface of the plates had dried, 6mm sterile paper disks were placed on top using sterile forceps. 10µL of 3% hydrogen peroxide was placed on disks, and plates were incubated upright in the anaerobic chamber. The next day, a zone of clearing was noted around each disk, and its diameter was measured and recorded. A two-tailed t-test was used to determine if differences were significant.

O_2 Sensitivity

Strains were grown overnight in BHIS under anaerobic conditions. Aliquots of these starter cultures were used to inoculate 10ml of BHIS, and cultures were grown without shaking for several generations. When cultures reached mid-log phase, they were transferred to 15ml conical tubes in the anaerobic chamber. The tubes were capped, removed from the chamber, and centrifuged for 5 minutes at 5000rpm on a tabletop centrifuge. Supernatants were poured off, and the pellets were resuspended in 10mls oxygenated PBS (pH 7.2)+0.5% glucose+thymine. These suspensions were transferred to 125ml flasks stoppered with foam, and shaken at 600rpm and 37C under room air. Samples were taken over time and diluted in PBS. 10µl of diluted samples were spotted to BHIS plates that had been placed in the anaerobic chamber overnight to reduce. After approximately 24 hours, colony-forming units were counted under a stage microscope.

Results

Tpx and AhpC are major scavengers of low concentrations of hydrogen peroxide

Identification of endogenous sources of ROS requires inactivation of the major hydrogen peroxide scavenging enzymes. In a strain lacking such detoxifiers, H_2O_2 produced intracellularly can leak across the cell membrane and accumulate in the extracellular milieu, thus allowing for quantification (32). In *E. coli*, deletion of *ahpC* and two catalases, *katE* and *katG*, allowed for monitoring of H_2O_2 production by decreasing peroxide scavenging rates to less than 5% of wild-type (32). Because *B. fragilis* appears to encode only one catalase (*katB*), we hypothesized that a Δ*ahpC*Δ*katB* mutant should permit quantification of H_2O_2 production. While initial experimentation indicated that ROS production could be detected using this strain, the results were inconsistent and difficult to interpret. We wondered if this strain might still retain significant scavenging activity, thereby complicating our results. Indeed, analysis of H_2O_2 scavenging activity showed that this was the case (fig.1) With the Δ*ahpC*Δ*kat* double mutant as our starting background strain, we began deleting other putative peroxidases to test their effects on peroxide scavenging rates. While little difference was seen when additional mutations were made in *dyp*, *tps*, or *glp* (data not shown) deletion of *tpx* significantly reduced the rate of scavenging, resulting in a strain with approximately 20% of the peroxide scavenging activity of the wild-type (fig. 1). Deletion of *tpx* in a strain wild-type for *ahpC* and *katB*, however, showed no defect in H2O2 detoxification, a result consistent with previous studies (12). These results indicate that Tpx and AhpC are major

scavengers of low concentrations of hydrogen peroxide, as the role of catalase under such conditions is most likely minimal (32).

Deletions of ahpC and tpx do not impair detoxification of high concentrations of H_2O_2

Many bacteria employ a two-tiered approach to detoxification of hydrogen peroxide, with peroxidases scavenging micromolar concentrations while catalase reduces higher concentrations. This strategy is thought to preserve precious reductant when bacteria are faced with overwhelming peroxide stress, as the H_2O_2 decomposition mediated by catalase does not require NAD(P)H (32). However, there is some overlap in these approaches in *E. coli*, since mutating *ahpCF* in a *katG* strain lead to an increase in sensitivity to a high concentration (3%) of H_2O_2 compared to the catalase mutant alone (32). We therefore employed a disk diffusion assay to investigate the roles of the *B. fragilis ahpC* and *tpx* genes in protection against millimolar concentrations of peroxide. Comparisons of the zones of clearing (figure 2) indicated that none of the strains tested differed significantly in H_2O_2 sensitivity compared to the wild-type, including the Δ*ahpC*Δ*kat*Δ*tpx* mutant. These results highlight the multifaceted approach of *B. fragilis* to ROS detoxification and provide further insight into the aerotolerance of this bacterium.

AhpC and Tpx are important components of aerotolerance

Previous work has shown that mutations in *ahpC* increase the sensitivity of *B. fragilis* to room air, while a catalase mutation does not (30) . Figure 3 shows that wild-type *B. fragilis* exposed to 21% oxygen steadily loses viability over 4 days. Deletion of *tpx* increases the aerotolerance of the organism under these conditions, most likely by allowing for the upregulation of other detoxification enzymes. The Δ*ahpC*Δ*kat* strain is hypersensitive during the first 24 hours of exposure, at which point the rate of killing declines until colonies are no longer recovered at 4 days. However, the Δ*ahpC*Δ*kat*Δ*tpx* mutant is far more intolerant of room air, as viable cells are no longer detectable by 60 hours. This result further confirms the reduced peroxide scavenging activity demonstrated by this strain in figure 1, and highlights the importance of endogenously-generated ROS in the susceptibility of *B. fragilis* to oxygen.

Fumarate reductase is a major source of ROS when B. fragilis is exposed to oxygen

Bacterial strains hampered in peroxide scavenging ability accumulate H_2O_2, but because this species can cross cell membranes, these strains allow for extracellular ROS quantification (32). Additionally, because the strains employed in these assays encode superoxide dismutase (Sod), any superoxide formed is converted to H_2O_2. Therefore, both the enzymes mediating the two electron reduction of oxygen to hydrogen peroxide and those reducing O_2 univalently to superoxide are represented in the peroxide detection scheme detailed here.

Because the Δ*ahpC*Δ*kat*Δ*tpx* mutant was so significantly impaired in H_2O_2 scavenging activity, we were able to quantify ROS accumulation rates *in vivo* using this strain. When grown anaerobically, suspended in PBS+glucose at an OD_{600} of 0.1, and shaken in room air, this strain generated H_2O_2 at a rate of 36±6nM/min (fig. 4). This rate is significant considering that substantial damage to *E. coli* metabolism occurs even in the face of submicromolar H_2O_2 concentrations (26). We therefore attempted to identify the enzyme(s) responsible for the production of this toxic intermediate.

Endogenous sources of ROS have been identified in *E. coli* (20), and among these fumarate reductase (Frd) has been described as a potent generator of superoxide (14, 24). Under anaerobic conditions, Frd mediates the reduction of fumarate to succinate via a solvent-exposed flavin cofactor. Because Frd is membrane-localized, the reduction of fumarate can be coupled to the establishment of a proton gradient across the bacterium's inner membrane and can thereby lead to the production of ATP via an F-type ATP synthase (4). However, the flavin cofactor is readily oxidized by molecular oxygen and can therefore be a potent source of superoxide (14, 19) (fig. 4). The Imlay laboratory has suggested that this enzyme may play a large role in ROS formation in *Bacteroides thetaiotaomicron* due to the high electron flux through fumarate reductase during growth of this organism (25). With this in mind, we made a deletion of the *frdC* gene in the Δ*ahpC*Δ*kat*Δ*tpx* background to test whether loss of fumarate reductase activity significantly reduced the rate of ROS formation in *B. fragilis* challenged under room air. As illustrated in figure 5, the Δ*ahpC*Δ*kat*Δ*tpx*Δ*frdC* strain accumulated 19±1 nM

H_2O_2/min, indicating that fumarate reductase accounts for approximately 47% of the ROS made by air-exposed *B. fragilis*.

Deleting frdC from a superoxide dismutase mutant partially restores aerotolerance

Given that the *B. fragilis* fumarate reductase plays such a major role in ROS production, deleting *frdC* might increase aerotolerance. To test this, we constructed a Δ*sod*Δ*frdC* mutant and exposed it to room air. Superoxide dismutase has been shown to play a crucial role in protecting *B. fragilis* from oxidative damage by scavenging superoxide, as strains showing reduced Sod activity are hypersensitive to room air (28). As shown in figure 6, a Δ*sod* mutant quickly loses viability under these conditions. A nine-hour exposure results in a loss of viability greater than three orders of magnitude for this strain. However, the Δ*sod*Δ*frdC* strain shows reduced sensitivity, maintaining approximately 14% viability during this time period. No difference was seen during this time between wild-type and Δ*frdC* strains.

Discussion

Despite its classification as an anaerobe, studies of *B. fragilis* have indicated that the organism is capable of elaborating a complex and nuanced response to oxygen exposure. Much of the oxidative stress response is the result of global transcriptional changes mediated by OxyR (29, 34) and chief among these O_2-responsive genes are numerous peroxidases charged with the reduction and detoxification of hydrogen peroxide. While the destructive capability of H_2O_2 is well documented (16, 26), the apparent redundancy behind so many peroxidases is intriguing. This is especially true when considering that *B. fragilis* is thought to reside solely in the mammalian large intestine, an environment virtually oxygen-free (21). The presence of these peroxidase genes and the complex regulation of their transcription imply that they serve important and possibly distinct roles, but these roles have not been satisfactorily dissected.

In this work we have uncovered the important hydrogen peroxide scavenging function of the thioredoxin-dependent peroxidase, Tpx. Classified as a typical 2-Cys thiol specific antioxidant, this enzyme most likely draws electrons from reduced thioredoxin, which in turn is reduced by thioredoxin reductase (TrxB). While the enzyme's role in detoxification of organic peroxides has been previously documented (12), the same study showed that deleting *tpx* has no effect on sensitivity to H_2O_2 in an otherwise wild-type strain. Our results confirm this finding. However, Tpx plays a crucial role in H_2O_2 scavenging in a strain missing *ahpC* and *kat*, and contributes significantly to aerotolerance in this genetic background. This implies that the scavenging function of

Tpx is redundant if AhpC is present, although the source of reductant for these peroxidases differs. The flavoenzyme AhpF transfers electrons to AhpC from NADH, while Tpx receives reducing power from NADPH via thioredoxin reductase. By encoding peroxidases capable of tapping into different pools of reductant, *B. fragilis* stands ready to scavenge ROS quickly even if metabolic requirements should temporarily deplete certain electron sources.

The discovery that fumarate reductase is a major source of reactive oxygen species in air-exposed *B. fragilis* was not entirely surprising, as this enzyme was found to produce ROS in anaerobically-grown *E. coli* shaken in room air (20), and *in vitro* studies of Frd in the closely-related organism *B. thetaiotaomicron* have indicated that this enzyme generates superoxide in a manner similar to the *E. coli* enzyme (25) . However, our results represent the first successful attempt at quantifying *in vivo* ROS generation in a bacterium classified as an "anaerobe", and as such should allow for greater insights into the fundamental differences between bacteria that can grow in an oxygenated environment and those that cannot. Interestingly, previous work has shown that many of the TCA cycle genes of *B. fragilis* are up-regulated 5-28-fold when the organism is aerated, ostensibly for the generation of reductants necessary for maintaining a reduced cytoplasm (34). However, transcription of *frd* is down-regulated approximately 3-fold, perhaps suggesting that an adaptive response is taking place to reduce ROS generation. This reduction in transcription occurs in an *oxyR* mutant as well as wild-type (34), so if it is the result of active repression, *B. fragilis*'s global transcriptional response to oxidative stress is even more sophisticated than previously thought. Alternatively, the reduction of

frd mRNA could be linked to a decrease in fumarate availability due to the ROS-mediated destruction of fumarase, the enzyme responsible for the production of this intermediate (25). Such regulation has been shown to be co-ordinated by the transcriptional factor FNR in *E. coli* (17, 18), and two such regulators have been annotated as FNRs in *B. fragilis*.

Work with the *E. coli* fumarate reductase demonstrated that the enzyme produces significantly less ROS *in vivo* in the presence of cytochrome oxidase (20), presumably by a back-oxidation of Frd by the quinone pool, ultimately depositing the electrons onto cytochrome oxidase rather than directly onto oxygen. The authors suggest that cytochrome oxidases in anaerobes might act similarly, thus limiting the rate of ROS production when cells are aerated. *B. fragilis* encodes such a cytochrome oxidase (3), implying that the Frd-mediated ROS generation rates in this study may increase in a *cyd* mutant. This remains to be tested.

Overall, these results have helped to define the metabolic cycle of reactive oxygen species in *Bacteroides*. Further studies in anaerobes should help to more clearly characterize the spectrum of oxygen tolerance displayed by all microbes.

Acknowledgments

We are grateful to Carol Kumamoto for assistance with the fluorometric studies.

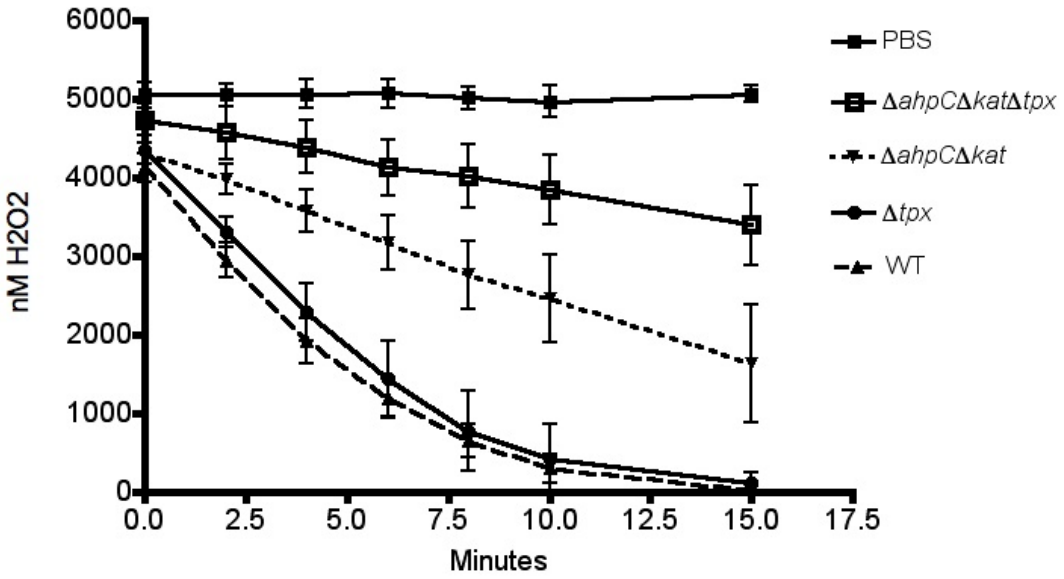


Figure 1. AhpC and Tpx have important and partially redundant H_2O_2-scavenging roles in *B. fragilis*. Cells grown in BHIS were washed and resuspended in PBS to OD_{600}=0.1. 5µM H_2O_2 was added to suspensions to start the assay and samples were taken over time. After a brief centrifugation, supernatants were assayed for [H_2O_2] using the Amplex Red protocol. PBS (black boxes), ADB77 (triangles), BM50 (upside-down triangles), BM95 (circles), BM105 (open boxes). Shown are the average rates of peroxide scavenging for three experiments ±SEM.

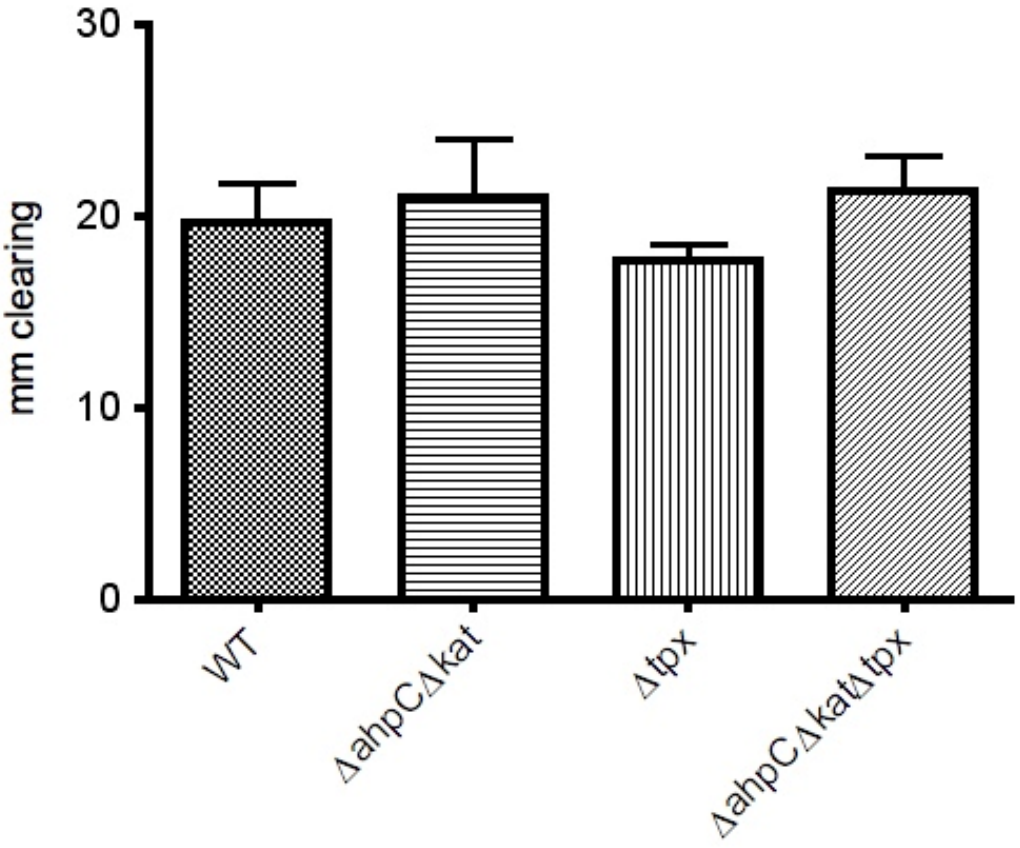


Figure 2. AhpC and Tpx do not contribute significantly to detoxification of high concentrations of exogenous hydrogen peroxide. Zones of clearing around 6mm paper disks soaked with 10µl 3% H_2O_2 were measured. Shown are the averages of 3 replicates ±SEM.

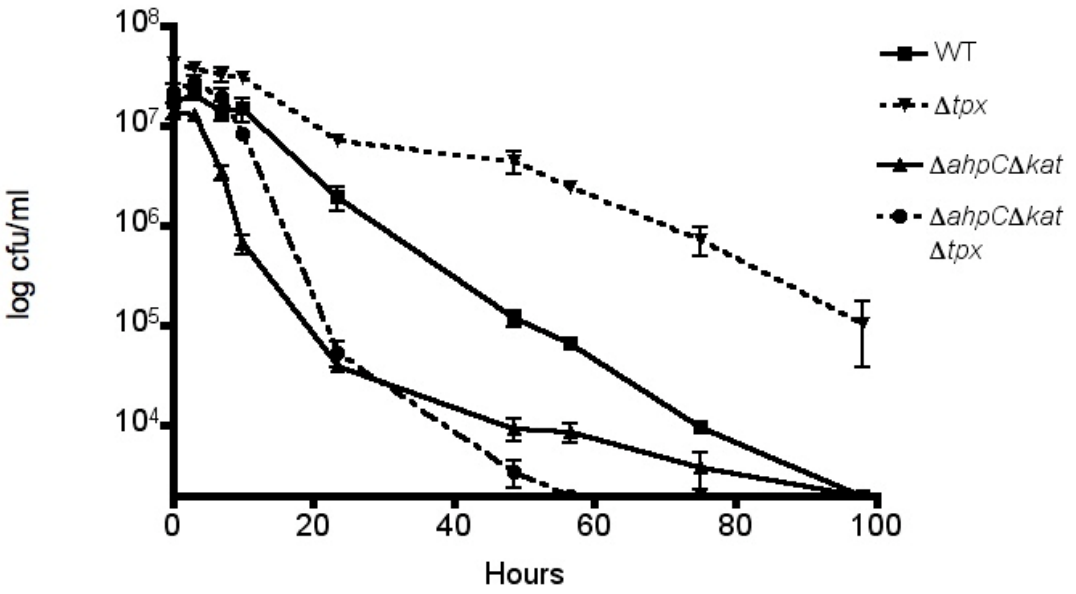


Figure 3. A *B.fragilis* strain missing AhpC, Tpx, and Kat is hypersensitive to 21% oxygen. Anaerobically-grown cells were suspended in PBS+0.5%glucose+50µg/ml thymine and shaken in room air at 37°C. Samples were taken over time, diluted, and plated on pre-reduced BHIS in anaerobic chamber. Colony-forming units were determined after approximately 24 hours. ADB77 (boxes), BM50 (triangles), BM95 (upside-down triangles), BM105 (circles). Shown are the averages of three experiments ±SEM.

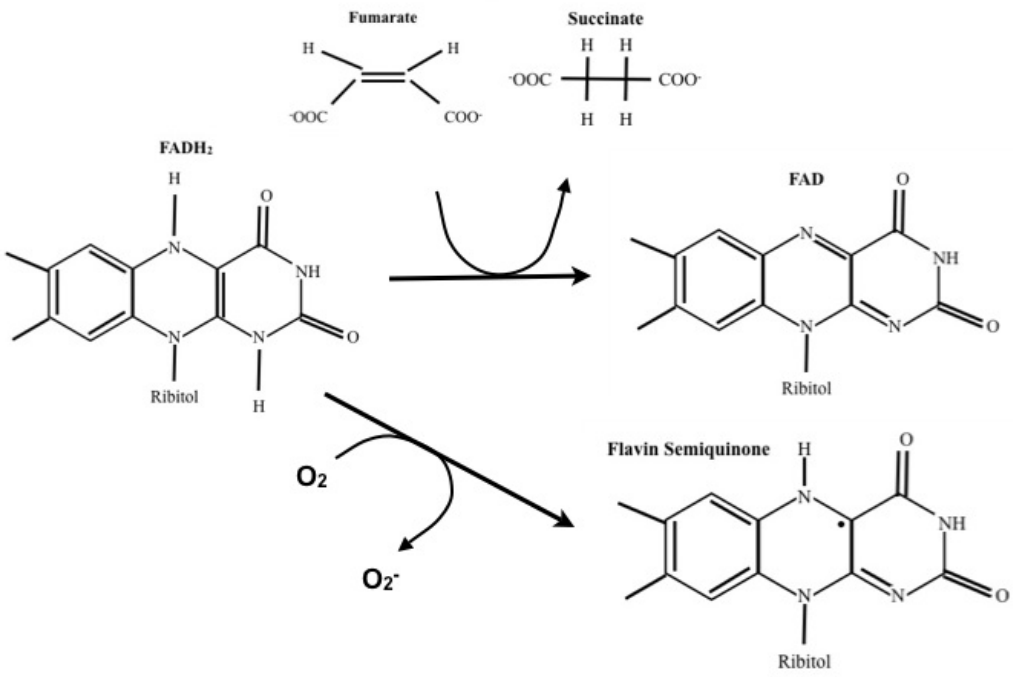


Figure 4. Potential reactions mediated by the exposed flavin associated with fumarate reductase. Electrons carried by $FADH_2$ can be donated to fumarate to produce succinate and the fully oxidized FAD. Should oxygen come into contact with $FADH_2$, however, one electron can be transferred to give superoxide and the partially reduced flavin semiquinone, which can be stabilized due to the aromatic ring structure.

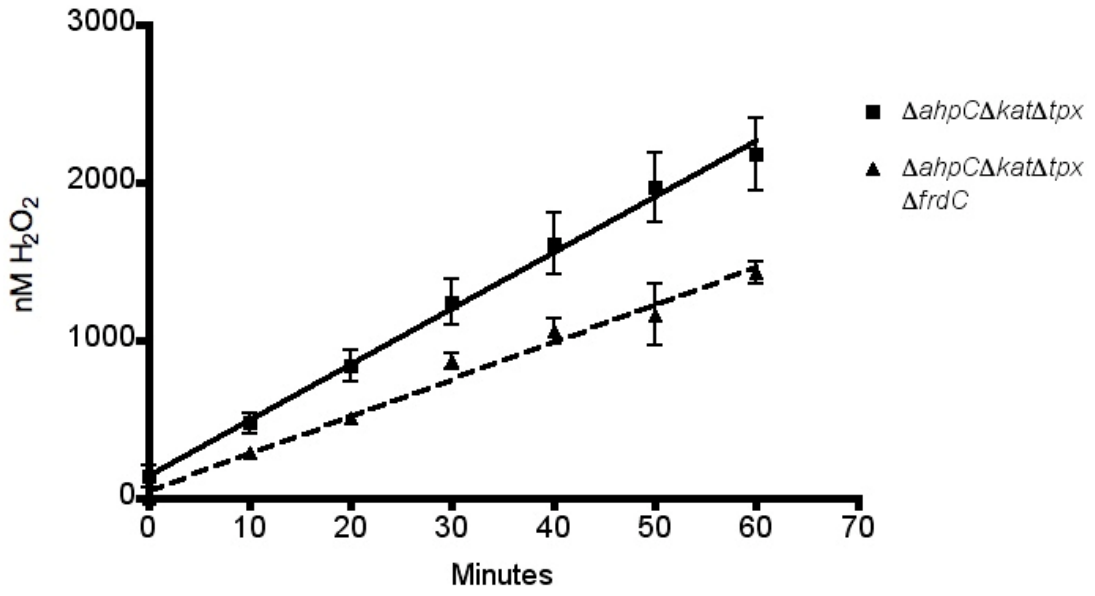


Figure 5. Fumarate reductase is a major source of ROS in *B. fragilis*. Cells grown to log-phase were centrifuged, resuspended in AMM lacking cysteine, and shaken for 1 hour at 37°C under room air. Cells were again centrifuged, washed, and resuspended in PBS+thymine to an OD_{600}=0.1. Freshly-prepared glucose was added to 0.05% just before start of assay. Suspensions were shaken at 37°C under room air throughout the course of the assay. Samples were taken over time and cells were centrifuged. Supernatants were assayed for [H_2O_2] using the Amplex Red protocol. BM105 (squares), BM112 (triangles). Shown are the averages of three assays ± SEM.

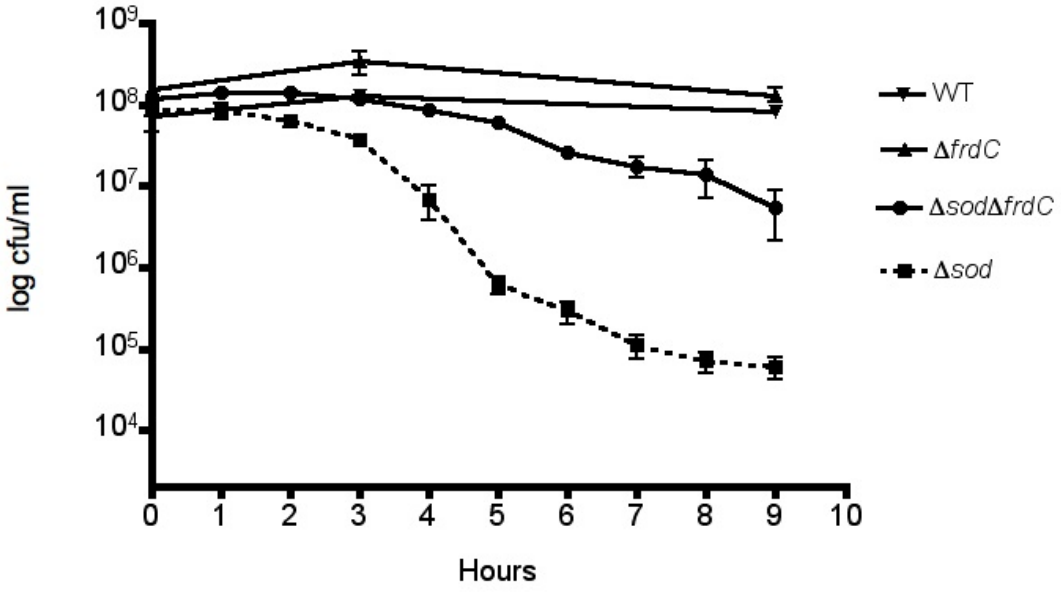


Figure 6. Deleting *frdC* partially protects a Δ*sod* mutant from O_2-dependent killing. Anaerobically-grown cells were suspended in PBS+glucose+thymine and shaken in room air at 37°C. Samples were taken over time, diluted, and plated to pre-reduced BHIS. Colony-forming units were enumerated after 24-48 hours. ADB77 (upside-down triangles), ADB247 (triangles), MBD616 (boxes), ADB247/616 (circles). Shown are the averages of three experiments ±SEM.

References

1. **Baughn, A. D., and M. H. Malamy.** 2003. The essential role of fumarate reductase in haem-dependent growth stimulation of *Bacteroides fragilis*. Microbiology **149:**1551-1558.
2. **Baughn, A. D., and M. H. Malamy.** 2002. A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle. Proc Natl Acad Sci U S A **99:**4662-4667.
3. **Baughn, A. D., and M. H. Malamy.** 2004. The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. Nature **427:**441-444.
4. **Biel, S., J. Simon, R. Gross, T. Ruiz, M. Ruitenberg, and A. Kroger.** 2002. Reconstitution of coupled fumarate respiration in liposomes by incorporating the electron transport enzymes isolated from *Wolinella succinogenes*. Eur J Biochem **269:**1974-1983.
5. **Cunningham, L., M. J. Gruer, and J. R. Guest.** 1997. Transcriptional regulation of the aconitase genes (acnA and acnB) of *Escherichia coli*. Microbiology **143 (Pt 12):**3795-3805.
6. **Djaman, O., F. W. Outten, and J. A. Imlay.** 2004. Repair of oxidized iron-sulfur clusters in *Escherichia coli*. J Biol Chem **279:**44590-44599.

7. **Fridovich, I.** 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? Ann N Y Acad Sci **893:**13-18.

8. **Fridovich, I.** 1998. Oxygen toxicity: a radical explanation. J Exp Biol **201:**1203-1209.

9. **Godoy, V. G., M. M. Dallas, T. A. Russo, and M. H. Malamy.** 1993. A role for *Bacteroides fragilis* neuraminidase in bacterial growth in two model systems. Infect Immun **61:**4415-4426.

10. **Guiney, D. G., P. Hasegawa, and C. E. Davis.** 1984. Plasmid transfer from *Escherichia coli* to *Bacteroides fragilis*: differential expression of antibiotic resistance phenotypes. Proc Natl Acad Sci U S A **81:**7203-7206.

11. **Hanahan, D., J. Jessee, and F. R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol **204:**63-113.

12. **Herren, C. D., E. R. Rocha, and C. J. Smith.** 2003. Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. Gene **316:**167-175.

13. **Imlay, J. A.** 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem **77:**755-776.

14. **Imlay, J. A.** 1995. A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. J Biol Chem **270:**19767-19777.

15. **Imlay, J. A.** 2003. Pathways of oxidative damage. Annu Rev Microbiol **57:**395-418.

16. **Jang, S., and J. A. Imlay.** 2007. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J Biol Chem **282:**929-937.

17. **Jones, H. M., and R. P. Gunsalus.** 1987. Regulation of *Escherichia coli* fumarate reductase (frdABCD) operon expression by respiratory electron acceptors and the fnr gene product. J Bacteriol **169:**3340-3349.

18. **Jones, H. M., and R. P. Gunsalus.** 1985. Transcription of the *Escherichia coli* fumarate reductase genes (frdABCD) and their coordinate regulation by oxygen, nitrate, and fumarate. J Bacteriol **164:**1100-1109.

19. **Korshunov, S., and J. A. Imlay.** 2006. Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. J Bacteriol **188:**6326-6334.

20. **Korshunov, S., and J. A. Imlay.** 2010. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. Mol Microbiol **75:**1389-1401.

21. **Leavitt, M.** 1970. Oxygen Tension in the Gut. New England Journal of Medicine **282:**1039-1040.

22. **Liochev, S. I., and I. Fridovich.** 1993. Modulation of the fumarases of *Escherichia coli* in response to oxidative stress. Arch Biochem Biophys **301:**379-384.

23. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem **244:**6049-6055.

24. **Messner, K. R., and J. A. Imlay.** 2002. Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. J Biol Chem **277:**42563-42571.

25. **Pan, N., and J. A. Imlay.** 2001. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. Mol Microbiol **39:**1562-1571.

26. **Park, S., X. You, and J. A. Imlay.** 2005. Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of *Escherichia coli*. Proc Natl Acad Sci U S A **102:**9317-9322.

27. **Parsonage, D., P. A. Karplus, and L. B. Poole.** 2008. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. Proc Natl Acad Sci U S A **105:**8209-8214.

28. **Privalle, C. T., and E. M. Gregory.** 1979. Superoxide dismutase and O_2 lethality in *Bacteroides fragilis*. J Bacteriol **138:**139-145.

29. **Rocha, E. R., G. Owens, Jr., and C. J. Smith.** 2000. The redox-sensitive transcriptional activator OxyR regulates the peroxide response regulon in the obligate anaerobe *Bacteroides fragilis*. J Bacteriol **182:**5059-5069.

30. **Rocha, E. R., T. Selby, J. P. Coleman, and C. J. Smith.** 1996. Oxidative stress response in an anaerobe, *Bacteroides fragilis*: a role for catalase in protection against hydrogen peroxide. J Bacteriol **178:**6895-6903.

31. **Rocha, E. R., and C. J. Smith.** 1999. Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. J Bacteriol **181:**5701-5710.

32. **Seaver, L. C., and J. A. Imlay.** 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. J Bacteriol **183:**7173-7181.

33. **Sund, C. J., W. Greg Wells, and C. Jeffrey Smith.** 2006. The *Bacteroides fragilis* P20 scavengase homolog is important in the oxidative stress response but is not controlled by OxyR. FEMS Microbiol Lett **261:**211-217.

34. **Sund, C. J., E. R. Rocha, A. O. Tzianabos, W. G. Wells, J. M. Gee, M. A. Reott, D. P. O'Rourke, and C. J. Smith.** 2008. The *Bacteroides fragilis* transcriptome response to oxygen and H_2O_2: the role of OxyR and its effect on survival and virulence. Mol Microbiol **67:**129-142.

35. **Tang, Y. P., M. M. Dallas, and M. H. Malamy.** 1999. Characterization of the Batl (Bacteroides aerotolerance) operon in *Bacteroides fragilis*: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in *in vivo* model systems. Mol Microbiol **32:**139-149.

36. **Thompson, J. S., and M. H. Malamy.** 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from *Bacteroides fragilis* TAL2480 reveals strong similarity between CfiA and *Bacillus cereus* beta-lactamase II. J Bacteriol **172:**2584-2593.

37. **Varel, V. H., and M. P. Bryant.** 1974. Nutritional features of *Bacteroides fragilis subsp. fragilis.* Appl Microbiol **28:**251-257.

38. **Varghese, S., Y. Tang, and J. A. Imlay.** 2003. Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. J Bacteriol **185:**221-230.

39. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine

methylation in plasmid and phage recombinants. Nucleic Acids Res **17:**3469-3478.

CHAPTER 2

Characterization of *Bacteroides fragilis* Mutants That Grow Under Increased Oxygen

Abstract

Bacteroides fragilis is capable of robust growth in environments containing up to 0.05% atmospheric oxygen, but higher concentrations prevent proliferation by an unknown mechanism. In this report we describe the isolation of *B. fragilis* mutants that grow under microaerobic conditions (0.2-2% oxygen). This "oxygen-enabled" phenotype is not due to upregulation of the oxidative stress response mediated by the transcriptional factor OxyR, as a strain encoding a constitutively active allele of OxyR was not oxygen-enabled nor did it give rise to an increased frequency of enabled colonies. Additionally, strains lacking catalase (*katB*), superoxide dismutase (*sod*), cytochrome oxidase (*cyd*), or the Bacteroides aerotolerance operon (*bat*) gave rise to O_2-enabled colonies at frequencies similar to wild-type, indicating that the phenotype does not require all of the elements of the oxidative stress response. We have determined that these oxygen-enabling spontaneous and transposon-insertion mutations occur in a previously uncharacterized *orf* that we designate *oxe*. The Oxe protein is predicted to encode a flavoprotein oxidoreductase. We isolated an in-frame, internal deletion of the *oxe* gene which exhibited the oxygen-enabled phenotype, and this mutant could be re-sensitized to oxygen by providing a wild-type copy of *oxe* on a plasmid *in trans*. Microaerobic growth of the Δ*oxe* mutant was characterized by an auxotrophy for several amino acids, including isoleucine, valine, threonine, methionine, and cysteine. By deleting catalase (*kat*), alkylhydroperoxide reductase (*ahpC*), and thioredoxin-dependent peroxidase (*tpx*) from the wild-type and the Δ*oxe* mutant, we were able to measure the rates of endogenous production of hydrogen peroxide when these strains were exposed to room

air. The Δ*oxe* strain produced peroxide at a slightly faster rate than wild-type. After induction of the oxidative stress response, the Δ*oxe* strain scavenged exogenous H_2O_2 at a rate about three times that of the wild-type. We suggest that the oxygen-enabled phenotype is most likely due to increased detoxification of this reactive oxygen species.

Introduction

When cyanobacteria developed the ability to strip electrons from water approximately 2.5 billion years ago, they set in motion a series of environmental changes that would have profound effects on the evolution of life on this planet. The release of molecular oxygen (O_2) as a waste product of photosynthesis would sabotage the delicate metabolic circuitry utilized by ancient microbes for energy production and leave these organisms vulnerable to DNA damage and ultimately death. This short-circuiting resulted from the adventitious partial reduction of O_2 by microbial enzymes and transition metals. Free iron (14, 17) and flavin-containing proteins (24, 27, 28, 31, 50), key components of the metabolic networks employed by these organisms, can donate electrons univalently to oxygen to produce superoxide (O_2^-), or sequentially to give rise to hydrogen peroxide (H_2O_2). These partially reduced derivatives of oxygen, known as reactive oxygen species (ROS), are capable of wreaking havoc on cellular machinery, causing the destruction of important iron sulfur clusters, oxidation and inactivation of key metabolic enzymes, and DNA lesions (1, 6, 7, 13, 19-22). Primordial microbes facing extinction in this newly-oxygenated environment were forced to retreat to protected niches in reducing sediments or to evolve mechanisms of protection from oxidative stress. These defenses included the elaboration of ROS detoxifying enzymes like superoxide dismutase (Sod) (29), catalases and peroxidases (18), as well as metabolic enzymes fortified against ROS damage (26, 46, 47). With such protective measures in place, these bacteria could not only grow

despite the presence of oxygen, but could further evolve to harness the enormous energy-generating potential of this powerful electron acceptor.

Anaerobic bacteria cannot replicate in the presence of oxygen and may have evolved from those sediment-dwelling ancient microbes. For this reason, obligate anaerobiosis was once thought to be rooted in the lack of ROS detoxifying enzymes (30). Extensive evidence to the contrary has since negated this hypothesis, but the molecular mechanisms underlying anaerobiosis have remained elusive. While the possibility remains that molecular oxygen itself can cause growth cessation in this class of organisms (33), a complete picture of O_2 sensitivity has not yet taken shape.

B. fragilis provides a good model for testing theories of oxygen sensitivity. Previously classified as a "strict anaerobe," this mammalian commensal has since been found to grow in and benefit from nanomolar concentrations of oxygen (4). And while it cannot replicate in room air, this organism is capable of mounting a strong response to aeration (35, 42) that results in the production of numerous ROS scavenging enzymes (14, 34, 37, 39-41) and other factors (43) that contribute to its impressive aerotolerance. *B. fragilis*'s unique position in the oxygen tolerance spectrum has allowed us to gain valuable insights into the nature of anaerobiosis. Herein we characterize derivatives of this bacterium that, due to mutation in a single gene (*oxe*), are capable of growth in up to 1.5% oxygen, representing a 30-fold increase over wild-type *B. fragilis*. Additionally, an *oxe* mutant is even more tolerant of room air than its wild-type counterpart, thus providing an intriguing link between those bacteria that thrive in aerobic environments and those that do not.

Materials and Methods

Reagents

Hydrogen peroxide and horseradish peroxidase were purchased from Sigma and Amplex Red was obtained from Invitrogen.

Growth conditions

Anaerobiosis was maintained by using a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. *B. fragilis* was grown in brain heart infusion broth supplemented with 0.5% yeast extract and 15µg/ml hematin (BHIS) or anaerobic minimal medium (AMM) containing 0.5% glucose, as previously described (3). In some cases super anaerobic minimal medium (SAMM) plates containing 150µg/ml hemin were used (45). For all *thyA* mutants, thymine was added to 50µg/ml. Gentamicin (50µg/ml), rifampicin (50µg/ml), trimethoprim (80µg/ml) and tetracycline (2.5µg/ml) were added to plates where appropriate. *E. coli* was grown aerobically in L broth, and chloramphenicol (25µg/ml), ampicillin (100µg/ml), and tetracycline (10µg/ml) were added where appropriate.

Strains and plasmids

E. coli strain DH5α was used for cloning, and strain HB101/pRK231 was used for mobilization of plasmids from DH5α to *B. fragilis* recipient strains. DH5α was made competent for transformation through use of the RbCl method previously described (12).

Strain	Relevant characteristics	Reference
B. fragilis		
ADB77	TM4000Δ*thyA*, Tp^R	(3)
ADB267	ADB77Δ*oxe*	this work
ADB266	ADB77Δ*cydAB*	(4)
IB260	638RΔ*kat*::Tet^R	(37)
IB263	638R*oxyR*^{c}	(38)
MBD616	TM4000 *thyA*$_2$Δ*sodA*	lab strain
ADB267/616	ADB77Δ*oxe*Δ*sod*	this work
YT135	TM4000 *batD*::Tn4400'	(43)
BM105	ADB77Δ*kat*Δ*ahpC*Δ*tpx*	this work
BM104	ADB267Δ*kat*Δ*ahpC*Δ*tpx*	this work
E. coli		
DH5∝	λ nonlysogen	(49)
HB101	*rpsL20*, host strain for pRK231	(44)

Plasmids		
pRK231	RP4 derivative, Tet^R, Tra^+	(9)
pADB242	pYT102 derivative, 0·35 kb *Bam*HI–*Hin*dIII fragment replaced by 18 bp *Bam*HI–*Hin*dIII fragment from pCR2.1-TOPO (Invitrogen) Cm^R	(2)
pADB267	pADB242Δ*oxe*	this work
pADB293	pJST61*oxe*$^+$	this work
pJST61	*B. fragilis-E. coli* shuttle vector, Erm^R, Amp^R	(44)

Table 1. Bacterial strains and plasmids used in this study

DNA manipulation

Primers used in this study are listed in Table 2. Genomic sequence for *B. fragilis* NCTC9343 was provided by the Pathogen Sequencing Group at the Sanger Centre (www.sanger.ac.uk/Projects/B_fragilis) or by the Pedant3 webpage (http://pedant.gsf.de). Oligonucleotide primers were synthesized by IDT (Iowa City, IA). *In silico* analysis of DNA and protein sequences was performed using DNA STRIDER 1.2 (DNAstar, Madison, WI) and MACVECTOR 7.0 (Oxford Molecular, Madison, WI). Genomic DNA was amplified using HotStarTaq Master Mix (Invitrogen, Carlsbad, CA). Plasmid

and PCR product purifications were performed with QIAprep spin columns (Qiagen, Chatsworth, CA). Where indicated, DNA was digested with restriction enzymes purchased from New England Biolabs (Beverly, MA). Ligations were performed using T4 DNA Ligase from Invitrogen (Carlsbad, CA). Primers abn01 and abn04 were used in combination with primer 646J to amplify *oxe*::IS4400 junction fragments by using the PCR method. DNA sequencing was performed with the corresponding primers by the Tufts University Nucleic Acids and Protein Core Facility.

Primer number	Region of homology	Sequence
61RAB	pADB242	GGCGCGCCGTAAGGAAAGTGGCTCTCAG
646J	Tn4400’	GACTTGGATACCTCACGC
B. fragilis		
abr1	*orf* upstream of *oxe*	GAAGAAGCTTTGAGTGTGGACGG
abr2	*oxe*	GGGCCATGGGCCACATTCCCTCAAAGAGG
abr3	*oxe*	AGACCATGGCACGTGCTATGGCC
abr4	*nagB*	TGCGGATCCAGGTGATCTGCTTCG
abr5	*orf* upstream of *oxe*	GCAATGACTGCGGATCGAGGTG
abr6	*orf* upstream of *oxe*	CCTGGGATCCGATCTGATACTTGAGCG

abr7	*nagB*	CGCCGGATCCGAAACGGACTGATAGTCCG
abn01	*oxe*	GAAGAAGCTTTGAGTGTGGACGG
abn04	*oxe*	TGCGGATCCAGGTGATCTGCTTCG
B. thetaiotaomicron		
BAM110	*orf* upstream of *oxe*	AAGAATGAAAGAAAGGGGAGC
BAM111	*oxe*	GAATCCTTCAATCATGCCGAA
BAM112	*oxe*	GGAGGACTGCCTATCACTACC
BAM113	*nagB*	AATGTGCAGCCCACTGAGATA

Table 2. Primers used in this study

Strain construction

All deletion mutants were constructed using a double-crossover technique previously described (11). In order to build a deletion construct for *oxe*, a fragment consisting of 724bp of *oxe* upstream sequence (including a portion of the upstream *orf*) and 86bp of *oxe* amino-terminal coding sequence was amplified using primers abr1 and abr2. The downstream fragment was made by PCR amplifying a region consisting of 44bp of *oxe* carboxy-terminal coding sequence and 836bp of downstream sequence (including a portion of *nagB*). After purifying PCR products, the upstream fragment was digested

with HindIII and NcoI, while the downstream fragment was digested with BamHI and NcoI. These digested products were purified and ligated via a three-way reaction to pADB242 that had been digested with BamHI and HindIII to create pADB267.

The suicide plasmid pADB267 was delivered to the recipient *B. fragilis* strains as previously described (44). Tetracycline-resistant colonies were screened for the appropriate cointegrant event using primers 61RAB and abr5. Isolates demonstrating recombination at the *oxe* locus were grown overnight in BHIS with thymine to allow for recombination events leading to resolution of the disrupting plasmid. The presence of the *thyA* gene on pADB242 sensitizes cointegrants to trimethoprim, while those resolvants that have excised the plasmid are trimethoprim-resistant. For this reason, the culture was plated to SAMM containing glucose, thymine, and trimpethoprim. Colonies arising after 3-4 days were purified and then screened for tetracycline-sensitivity on BHIS+thymine plates. Tetracycline-sensitive colonies were then used as template in a PCR with primers roo5 and roo4 to identify Δ*oxe* clones.

The *oxe* complementing plasmid was created by PCR amplifying a region from 270bp upstream of the *oxe* translational start site to 77bp downstream of the *oxe* coding sequence using primers roo6 and roo7. The purified PCR product was digested with BamHI and ligated to BglII-digested pJST61 to create pADB293.

Growth curves

Dense cultures of wild-type and Δ*oxe* strains were sub-cultured as indicated into fresh medium. Where appropriate, Casamino acids were added to 200µg/ml. Cultures were grown at 37°C with shaking (200-300rpm) under anaerobic or microaerobic conditions (0.25-1% oxygen) as described below. Samples were taken over time, serially diluted in reduced PBS or growth medium, and 10µl aliquots were spotted on BHIS plates that had been reduced overnight in the anaerobic chamber. Colony-forming units were enumerated under a stage microscope after 1-2 days. Oxygen concentration was measured using the pyrogallol method (4) or with a Coy oxygen analyzer (model 630).

Efficiency of plating

B. fragilis strains were grown anaerobically to mid-log phase in BHIS. These cultures were serially diluted in PBS or medium and spread on BHIS plates that had been reduced in the anaerobic chamber overnight. Plates were transferred anaerobically to AnaeroPack boxes (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) fitted with side ports and sealed boxes were removed from anaerobic chamber. O_2 was injected through the side port to achieve the appropriate oxygen concentrations. Alternatively, plates were placed in an anaerobic chamber from which the O_2-scrubbing catalyst had been removed and the appropriate amount of oxygen had been added. Colony-forming units were enumerated after 3-5 days at 37°C. Efficiencies of plating were calculated by dividing the number of colonies formed under microaerobic conditions by the number of colonies formed anaerobically.

Oxygen challenge

Strains were grown overnight in BHIS under anaerobic conditions. These starter cultures were used to inoculate 10ml BHIS and grown for several generations until cells had reached mid-log phase. Cultures were centrifuged 10 minutes at 4000rpm and pellets were washed once with PBS. After a second centrifugation, pellets were resuspended in 10ml oxygenated PBS and 0.5% glucose and thymine were added. Samples were taken immediately, serially diluted, and a 10µl aliquot was spotted on pre-reduced BHIS plates (±Erm), which were incubated in the anaerobic chamber. Cell suspensions were transferred to 125ml foam-stoppered flasks and shaken at 600rpm and 37°C under room air. Samples were taken over time and processed as above. Colony-forming units were enumerated after 2 days of incubation.

H_2O_2 generation

Strains were grown for 2 days at 37°C in 5ml AMM. Aliquots of these dense cultures were then used to inoculate 10ml AMM. Cultures were shaken anaerobically at 100rpm in 50ml flasks and grown for several generations. When cultures had reached mid-log phase, they were transferred anaerobically to conical screw-cap tubes, sealed, and centrifuged 10 minutes at 4000rpm. Tubes were brought back into the anaerobic chamber and the supernatants were decanted. Cell pellets were resuspended in 10ml AMM lacking cysteine and transferred to 125ml foam-stoppered flasks. These flasks were shaken at 600rpm in room air at 37°C for one hour. Cultures were again transferred to conical screw-cap tubes and centrifuged as before. Cell pellets were washed twice with PBS and then resuspended in 20mls PBS+0.05% fresh glucose+thymine at an OD_{600} =0.1. Flasks were shaken at 600rpm in room air at 37°C. 1.1ml samples were drawn at

various time points as described above to determine H_2O_2 concentrations in the supernatants using Amplex Red as previously described (manuscript in preparation).

H_2O_2 scavenging

Strains were grown for 2 days at 37°C in AMM in the anaerobic chamber. These starter cultures were then used to seed 20ml AMM and were grown with shaking at 100rpm until mid-log phase. Cultures were then transferred to 50ml conical tubes and centrifuged 10 minutes at 4000rpm. Cell pellets were resuspended in 15mls AMM without cysteine and transferred to 125ml foam-stoppered flasks. These flasks were shaken 1 hour at 37°C under room air. Cultures were again centrifuged, washed twice with 15ml PBS, and resuspended in 15mls PBS. Cultures were adjusted to an OD_{600}=0.3 in 10mls PBS+thymine. Just before addition of H_2O_2, 50µl 10% freshly-prepared glucose was added.

A solution of H_2O_2 was made by diluting ~15µl 3%H_2O_2 into 1ml PBS. The OD_{240} was measured and the H_2O_2 concentration was calculated (ϵ=43.6$M^{-1}cm^{-1}$). A stock of 200µM was made by appropriate dilution of this solution. To begin a scavenging assay, 250µl of stock H_2O_2 was added to 10ml cultures in Hungate tubes to bring the starting concentration to 5µM. Assay was carried out as previously described (manuscript in preparation)

Results

Isolation of oxygen-enabled mutants

Recent work has shown that *B. fragilis* can grow under atmospheres containing up to 0.05% oxygen (500nM dissolved O_2). This growth is dependent on a membrane-localized cytochrome bd oxidase that couples O_2 reduction to energy generation (4). This high-affinity oxidase is saturated by 2µM oxygen, however, preventing increased growth yields when O_2 levels are raised (15) . Yet when wild-type *B. fragilis* was plated under microaerobic conditions (0.25-1.5%), colonies were found to form at a frequency of approximately 10^{-6} relative to an anaerobic control (table 3). Purification of these colonies revealed that they were capable of robust growth under microaerobic conditions, suggesting that they had acquired an increased resistance to oxidative damage.

B. fragilis encodes numerous oxidative stress defense enzymes that play a crucial role in protecting the organism from the damaging effects of ROS, and the expression of many of these proteins is controlled at the transcriptional level by OxyR (42). This tetrameric response regulator binds to specific sites within promoter regions, but under anaerobic conditions it cannot activate transcription. Introduction of ROS causes a conformational shift in OxyR that promotes interaction with RNA polymerase, leading to transcription of downstream genes (23, 32). Isolation of a mutant strain of *B. fragilis* encoding a constitutively-active OxyR (36) allowed us to test whether increased expression of genes involved in the oxidative stress response would allow for growth of this organism under

microaerobic conditions. Table 3 shows that this is not the case, as strain IB263 plated with the same efficiency as wild-type under 0.8% oxygen.

We also tested whether strains of *B. fragilis* that were unable to mount a full oxidative stress response (OSR) could give rise to oxygen-enabled colonies. Strains missing catalase, superoxide dismutase, cytochrome oxidase, or the Bacteroides aerotolerance operon (43) were found to give rise to enabled mutants with the same efficiency as wild-type under microaerobic conditions (table 3), suggesting that a fully-functional OSR was not necessary for this oxygen-enabled phenotype. One mutant, however, gave a surprising result. Deletion of a predicted flavoprotein oxidoreductase (bf0904) gave rise to a strain that plated with nearly 100% efficiency under microaerobic conditions (table 3). BLAST analysis suggested that this gene might encode a rubredoxin:oxygen oxidoreductase (Roo), an enzyme found to protect *Desulfovibrio vulgaris hildenborough* under 1% oxygen by preventing ROS formation (48). While the total homology between Oxe and Roo is 48%, our results suggested a different function for this gene product in *B. fragilis*, prompting us to name the locus *oxe* (*ox*ygen-*e*nabled) (fig. 1).

To determine if mutations in other genes could give rise to O_2-enabled strains, a pool of Tn4400' (43) transposon-insertion mutants was plated on BHIS under atmospheres containing up to 2% oxygen. A PCR-based screen was used to exclude isolates with Tn4400' insertions in *oxe*. Of 32 isolates that were analyzed, 23 showed insertions of the IS element from Tn4400' within *oxe* (A. Baughn, unpublished results). The other 9 were found to fall into two groups that had insertions that lay outside of the *oxe* locus, but PCR

amplification and sequencing of the *oxe* showed that each had acquired spontaneous mutations in *oxe* in addition to the transposon insertion (fig. 1A). Importantly, introduction of a plasmid encoding wild-type *oxe* into these strains prevented growth under microaerobic conditions, indicating that the O_2-enabled phenotype was due to the mutations in *oxe*. Additionally, four of the isolates that arose from microaerobic plating of the wild-type were sequenced across the *oxe* locus. All were found to contain mutations in *oxe* (ΔG673, ΔG1160, A245T (E82V), and C420G(stop)).

A Δoxe strain plates with the same efficiency under anaerobic and microaerobic conditions

Wild-type *B. fragilis* and the Δ*oxe* strain were grown anaerobically to mid-log phase in BHIS, serially diluted and spread on BHIS plates that had been reduced in the anaerobic chamber overnight. These plates were then incubated at 37°C anaerobically or in sealed boxes containing various concentrations of oxygen. After 5 days, colony-forming units were enumerated and compared. While both the wild-type and Δ*oxe* strains plated with approximately 100% efficiency in environments containing ≤0.05% oxygen compared to the anaerobic controls, they differed significantly in plating efficiencies at higher O_2 concentrations (fig. 2). Wild-type *B. fragilis* gave rise to colonies at a frequency between 10^{-6} and 10^{-7} under 0.2-2% oxygen and failed to form detectable numbers of colonies under more oxygenated environments. The Δ*oxe* strain, however, gave rise to similar numbers of colonies in all O_2 levels up to 2% (fig. 2), and plated with a frequency of approximately 10^{-6} under 5% oxygen. Importantly, when a plasmid-borne copy of *oxe*

was introduced into the Δ*oxe* strain, the increased plating efficiency was lost, indicating that the phenotype could be complemented (fig. 2).

A Δoxe strain grows in liquid medium under microaerobic conditions but is auxotrophic for certain amino acids

As shown in figures 3 and 4, wild-type *B. fragilis* and the Δ*oxe* strain grow equally well under anaerobic conditions in both rich and minimal medium, with doubling times of approximately 2 hours and 2.5 hours, respectively. However, when BHIS cultures are shaken under 0.5% oxygen, the Δ*oxe* mutant begins growing immediately, doubling approximately every 4 hours, while the wild-type enters a long lag phase lasting 20-30 hours (fig. 3). In AMM, neither wild-type nor the Δ*oxe* strain grow when shaken under microaerobic conditions, although supplementation with Casamino acids does restore growth to the Δ*oxe* mutant but not to the wild-type (fig.4 and data not shown). To further characterize the amino acid auxotrophy of the Δ*oxe* strain, we attempted to grow ADB267 in AMM under 0.5% oxygen with various combinations of individual amino acid supplements. The best combination for growth contained isoleucine, valine, threonine, methionine, and cysteine (fig. 5), although the growth rate with these additions did not match that found in fully supplemented medium.

Δoxe retains greater viability under room air than wild-type

While the Δ*oxe* mutant clearly demonstrated a growth advantage over the wild-type in a microaerobic environment, we wondered if the *oxe* deletion would increase aerotolerance when cells were shaken in room air. Figure 6 shows that the Δ*oxe* strain retains greater viability than the wild-type over several days of exposure, culminating in a ~10-fold advantage after 48 hours. The increased aerotolerance of the Δ*oxe* strain is lost when *oxe* is provided *in trans*, but introduction of an empty vector has no effect.

A strain lacking oxe accumulates H_2O_2 at a slightly faster rate than wild-type

oxe is predicted to encode a flavoprotein, a class of enzymes that utilize the flavins FAD or FMN as a cofactor in redox reactions. Electrons are funneled through this flavin moiety and onto a substrate. However, if the reduced flavin comes into contact with oxygen, the electrons can be transferred to O_2 to generate ROS (21, 22) . One model to explain the inability of wild-type *B. fragilis* to grow microaerobically, therefore, would posit that Oxe produces crippling amounts of ROS when cells are exposed to oxygen. To test this, we deleted *oxe* from a strain with deletions in catalase (*katB*), alkylhydroperoxide reductase (*ahpC*), and thioredoxin-dependent peroxidase (*tpx*). We have previously shown (manuscript in preparation) that this strain cannot effectively scavenge H_2O_2, so any peroxide made during exposure to oxygen can cross the cell membrane and be measured extracellularly using Amplex Red. Additionally, because this strain encodes Sod, half of all superoxide made will be converted to H_2O_2, so quantification of peroxide is a reflection of endogenous production of both reactive

oxygen species.

When the Δ*kat*Δ*ahpC*Δ*tpx* strain was shaken under room air, it accumulated H_2O_2 at a rate of 24±2nM/min, (fig. 7). However, deleting *oxe* from this genetic background resulted in a strain that accumulated 29±3nM H_2O_2/min, a rate slightly higher than the parent strain (fig. 7).

Δoxe strain scavenges H_2O_2 more efficiently than wild-type

In addition to AhpC and Tpx, there are four other peroxidases annotated in the *B. fragilis* genome, and it is possible that deleting *oxe* might activate them in some manner. We tested the H_2O_2 scavenging activity of wild-type and Δ*oxe* strains by first shaking them aerobically to induce expression of any ROS detoxification enzymes, and then adding exogenous peroxide to whole-cell suspensions normalized to an OD_{600}=0.3. Figure 8 shows that the Δ*oxe* mutant did indeed scavenge H_2O_2 more effectively than wild-type, with rates of 333±56nM/min and 119±18nM/min, respectively.

Spontaneous oxygen-enabled mutants arise in B. thetaiotaomicron

To test whether the oxygen-enabling phenomenon was specific to *B. fragilis* or could be recapitulated in other anaerobes, we attempted to isolate mutants of the closely-related organism *Bacteroides thetaiotaomicron* that were capable of growth under 0.5% oxygen. As described for *B.* fragilis above, we were able to isolate colonies of B. *thetaiotaomicron* ATCC 29741 capable of growth under 0.5% oxygen at a frequency of

~10^{-7} relative to anaerobic controls. *B. thetaiotaomicron* encodes a protein with 97.2% homology to Oxe, and the gene is surrounded by genes encoding a putative radical SAM-family protein and NagB as in *B. fragilis* (fig. 1B). Three colonies were purified on BHIS under 0.5% oxygen and the region around the *oxe* locus was sequenced following PCR amplification. Two of the three *oxe* genes had mutations (G578A ,G731T) relative to wild-type 29741, and these were predicted to lead to amino acid changes (R193H, S244I). The third isolate encoded a wild-type Oxe, but showed a predicted amino acid change (T322I) in the product of the *orf* upstream of *oxe*, possibly indicating that this putative radical SAM-family protein may play a role in an Oxe-dependent pathway.

Discussion

The discovery that *B. fragilis* can benefit from small amounts of oxygen (4) has led to even more questions regarding the inhibitory effect of oxygen on growth of some microbes. While many of these anaerobes can mount an oxidative stress response, there appear to be strict limits on exactly how much oxygen these organisms can tolerate. To gain insight into the nature of these barriers, we sought to isolate mutants of *B. fragilis* capable of growth under concentrations of oxygen restrictive to the wild-type.

We have demonstrated that mutants capable of growth under microaerobic conditions can be isolated at a frequency of ~10^{-6} relative to anaerobic controls, and that a fully functional oxidative stress response is not a prerequisite for isolation of these O_2-enabled strains. All of the oxygen-enabled mutants studied to date have lesions in a previously uncharacterized gene we have named *oxe*. BLAST analysis of the predicted Oxe protein indicates that it bears some homology to rubredoxin:oxygen oxidoreductase (Roo), an enzyme shown to be important in the response of *Desulfovibrio vulgaris hildenborough* to microaerobic conditions. A mutation in *roo* was shown to sensitize this organism to 1% oxygen (48), so it is unlikely that Oxe functions as a Roo. Additionally, Oxe shows some homology to NorV, an anaerobic nitric oxide reductase found to protect *E. coli* from nitric oxide-mediated bacteriostasis (8). However, wild-type and Δ*oxe* were inhibited by similar concentrations of the nitric oxide-releasing chemical DEANONOate, and extensive amperometric studies suggested that these strains could reduce nitric oxide

at similar rates (data not shown). We therefore do not know the function of Oxe in the anaerobic physiology of *B. fragilis*. However, analysis of transposon mutants of *Bacteroides thetaiotaomicron* suggests that Oxe is essential for growth in the gut of gnotobiotic mice (10).

A Δ*oxe* strain did not demonstrate any growth defect relative to the wild-type under anaerobic conditions, indicating that Oxe is not essential for growth *in vitro*. In rich medium under microaerobic conditions, the Δ*oxe* mutant began growing immediately, while the wild-type lagged for over a day (fig. 3). The onset of growth at this late time point suggests that a variant population had overtaken the culture, and linear regression of the exponential growth rate suggests that this variant population arose from a few bacteria present at the beginning of the experiment. Following clonal purification under anaerobic conditions, isolates from this variant population were found to grow under microaerobic conditions, but not under room air. Therefore, the microaerobic growth seen for the ADB77 after 24 hours is most likely due to spontaneous *oxe* mutants overtaking the culture.

This Δ*oxe* mutant, however, showed an auxotrophy for amino acids when grown in a microaerobic environment. Isoleucine, valine, threonine, methionine, and cysteine partially alleviated this auxotrophy, indicating that the biosynthetic pathways for these amino acids had been damaged by aeration. Early work with an *E. coli sod* mutant demonstrated a similar conditional sensitivity to oxygen (5), a phenotype later found to be the result of superoxide-mediated destruction of the iron-sulfur cluster of

dihyrdroxyacid dehydratase (IlvD), a key enzyme in the production of branched-chain amino acids (25) . Additionally, it has been shown that methionine synthase is sensitive to diamide and high concentrations of H_2O_2 (17). Therefore, the auxotrophies displayed by the Δ*oxe* mutant under microaerobic conditions suggest that this strain is experiencing significant oxidative stress. The growth-stimulatory effect of cysteine is puzzling, for while this amino acid plays a key role in balancing the intracellular redox potential, the amount of cysteine already present in AMM (~4mM) should suffice for this purpose.

In addition to allowing microaerobic growth, deletion of *oxe* increased the tolerance of *B. fragilis* to room air. Interestingly, a strain encoding a constitutively active OxyR displayed neither of these characteristics (table 3 and (36)) despite its increased resistance to oxidative stress. This contrasts with studies with *Clostridium acetobutylicum* that showed that activation of its oxidative stress response via the deletion of the gene encoding the PerR repressor gave rise to a strain that grew slightly in room air and tolerated aeration much better than the wild-type (16). Obviously these distantly related anaerobes have different means for coping with oxygen.

We have shown that Oxe is not a major source of reactive oxygen species when *B. fragilis* is exposed to room air. If this enzyme is indeed a flavoprotein as BLAST analysis predicts, the flavin cofactor is most likely buried deep within the protein structure and thereby protected from autoxidation. This is in stark contrast to fumarate reductase (Frd), another flavin-containing enzyme that we have demonstrated accounts for approximately 47% of the ROS generated by aerated *B. fragilis* (manuscript in

preparation). Interestingly, a *frd* mutant does not grow microaerobically without an additional mutation in *oxe*, indicating that a substantial reduction in ROS production is not enough to give rise to an oxygen-enabled strain.

While deleting *oxe* did not lower the rate of production of endogenously-generated ROS, it did increase ROS detoxification. A Δ*oxe* strain scavenged a micromolar amount of H_2O_2 at a rate almost three times faster than the wild-type after the strains had been aerated to induce the oxidative stress response. The enhanced destruction of ROS most likely explains the oxygen-enabled phenotype, as an *oxe* mutant could more efficiently protect vulnerable metabolic enzymes under increased oxygen concentrations. The molecular mechanism behind this enhanced ROS detoxification is most likely not due to the increased expression of peroxide scavenging enzymes, for the strain encoding a constitutively-active OxyR did not allow for microaerobic growth. We therefore believe that the Δ*oxe* strain most likely accumulates excess reductant in the form of NAD(P)H, and that peroxidases can tap into this reductant pool to more efficiently scavenge H_2O_2. This hypothesis is perhaps supported by the slight increase in H_2O_2 production seen in the strain missing *oxe* (BM104), for an excess of NAD(P)H could be funneled into ROS-generating enzymes like fumarate reductase in this assay. Our model assumes that Oxe is an oxidoreductase, and that it reduces an intracellular substrate with electrons derived from these reduced dinucleotides. The identity of this substrate is currently unknown.

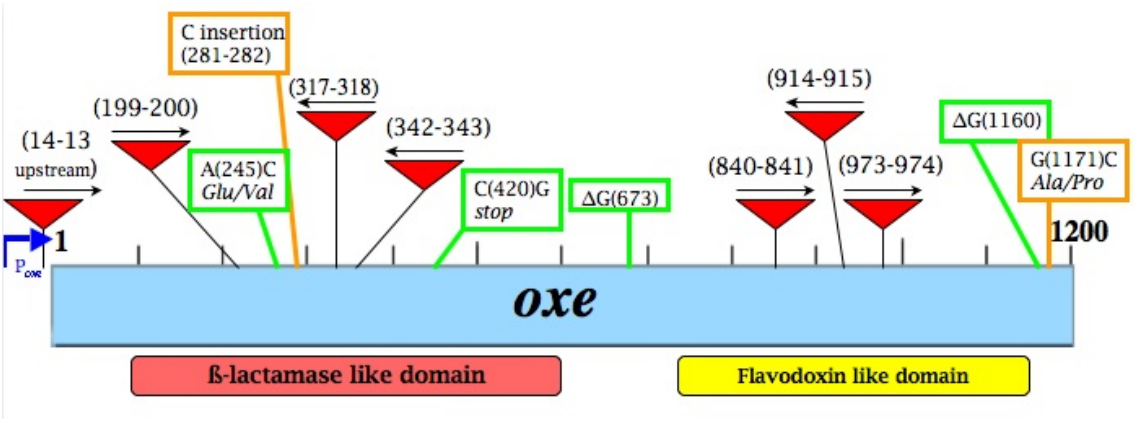
Work in *B. thetaiotaomicron* has demonstrated that exposure of this organism to room air causes the inactivation of multiple metabolic enzymes crucial to its fermentative lifestyle

(33). Surprisingly, pyruvate:ferreodoxin oxidoreductase (PFOR), a key enzyme in the fermentation of pyruvate, was shown to be specifically poisoned by molecular oxygen. This finding led the authors to speculate that the essentiality of such O_2-labile proteins to *Bacteroides* metabolism may be the reason these bacteria cannot grow aerobically. If this is true, the isolation of *B. thetaiotaomicron* mutants capable of microaerobic growth as described in this report would indicate that 1% oxygen is not enough to inactivate these critical enzymes. The first barrier to aerobic growth of *Bacteroides*, therefore, would appear to be ROS-mediated toxicity.

B. fragilis Strain	Relevant Characteristics	$\log_{10}$ (Efficiency of Plating)
ADB77	TM4000Δ*thyA*	-5.9
ADB267	ADB77Δ*oxe*	-0.03
ADB266	ADB77Δ*cydAB*	-6.3
IB260	638RΔ*katB*::tetQ	-5.6
IB263	638R*oxyR*^{C}	-6.3
MBD616	TM4000 *thyA*$_2$Δ*sodA*	-6.2
YT135	TM4000 *batD*::Tn4400’	-5.9

Table 3. Efficiencies of plating for various strains of *B. fragilis* under microaerobic conditions. Strains were grown anaerobically, serially diluted, and plated to BHIS. Some plates were transferred to boxes containing the indicated percentage of oxygen. After several days, colony-forming units were enumerated. Efficiency of plating is defined by the number of colonies arising in the presence of oxygen divided by the number of colonies arising anaerobically.

A.



B.

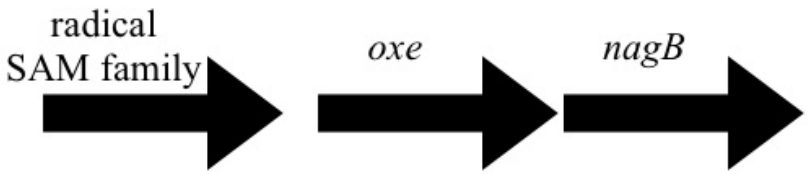


Figure 1. (A) Diagram of the *oxe* gene. The blue box represents the *oxe* gene. Predicted protein domains are shown below. Red arrows depict IS*4400*' insertions. Shown in the green boxes are the mutations found in spontaneous oxygen-enabled mutants of wild-type (ADB77) with nucleotide positions in parentheses. Orange boxes denote mutations found in oxygen-enabled mutants from transposon-insertion pool. (B) Genomic region surrounding the *oxe* gene. *nagB* = glucosamine 6-P deaminase.

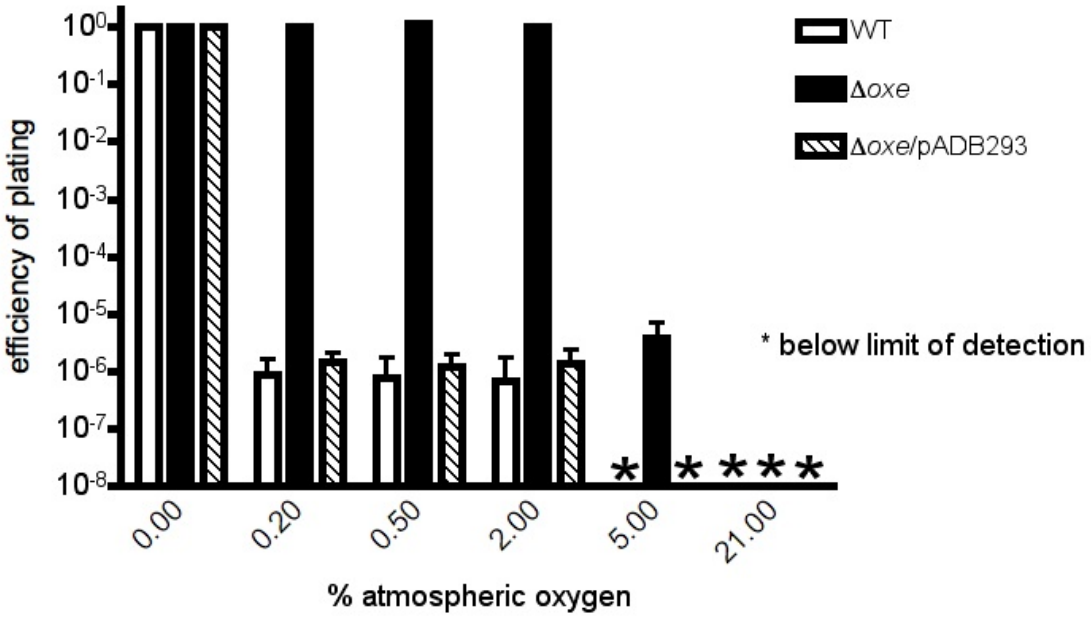


Figure 2. Efficiency of plating under various percentages of oxygen. ADB77 (clear bars), ADB267 (black bars), and ADB267 complemented with the *oxe* containing plasmid (hatched bars) were grown anaerobically and plated under different oxygen concentrations. Procedure and calculations are is in figure 1. (*A. Baughn, unpublished data*)

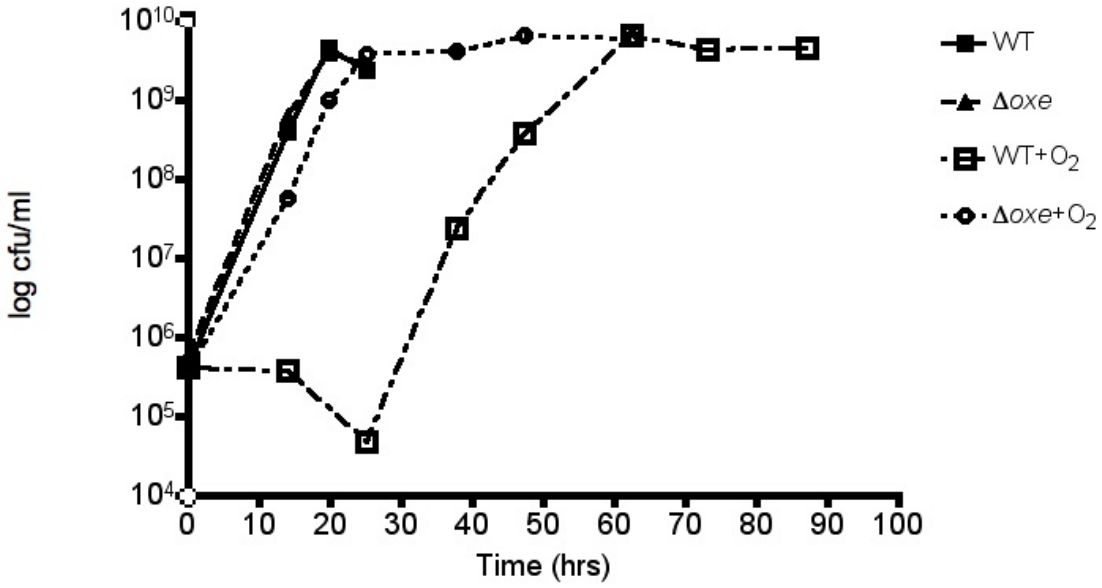


Figure 3. Growth of wild-type and Δ*oxe* in BHIS with and without 0.25% oxygen. Dense overnight cultures grown in BHIS were diluted 1:20000 into fresh medium. Cultures were grown at 37°C with shaking in anaerobic chamber or in chamber to which oxygen had been introduced. Samples were taken over time and serially diluted, and 10µl aliquots were spotted to BHIS plates that had been reduced overnight in the anaerobic chamber. Colony-forming units were enumerated after 1-2 days. Oxygen concentration in chamber was measured at the end of the experiment using the pyrogallol method and found to be 0.25%. ADB77 anaerobic (black boxes) and microaerobic (open boxes). ADB267 anaerobic (triangles) and microaerobic (circles). (*A. Baughn, unpublished data*)

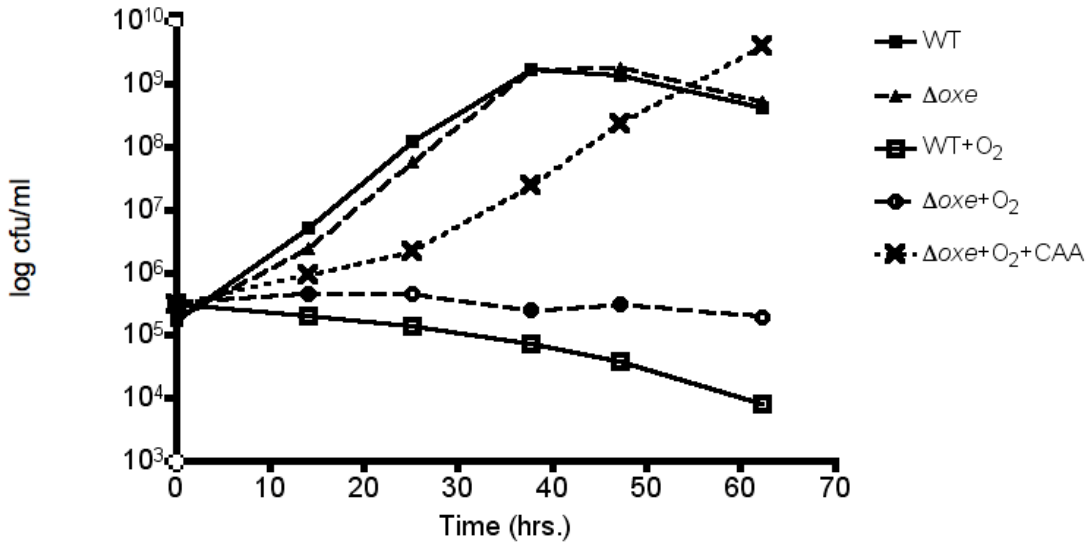


Figure 4. The Δ*oxe* strain is auxotrophic for amino acids when grown microaerobically. Strains were grown anaerobically in AMM until dense. Cultures were subbed into AMM and grown until mid-log phase, then subbed 1:20000 into fresh AMM. Where appropriate, case-amino acids were added to 200µg/ml. Cultures were grown at 37°C with shaking in anaerobic chamber or in chamber to which oxygen had been introduced. Samples were taken over time and serially diluted, and 10µl aliquots were spotted to BHIS plates that had been reduced overnight in the anaerobic chamber. Colony-forming units were enumerated after 1-2 days. Oxygen concentration in chamber was measured at the end of the experiment using the pyrogallol method and found to be 0.25%. (*A. Baughn, unpublished data).*

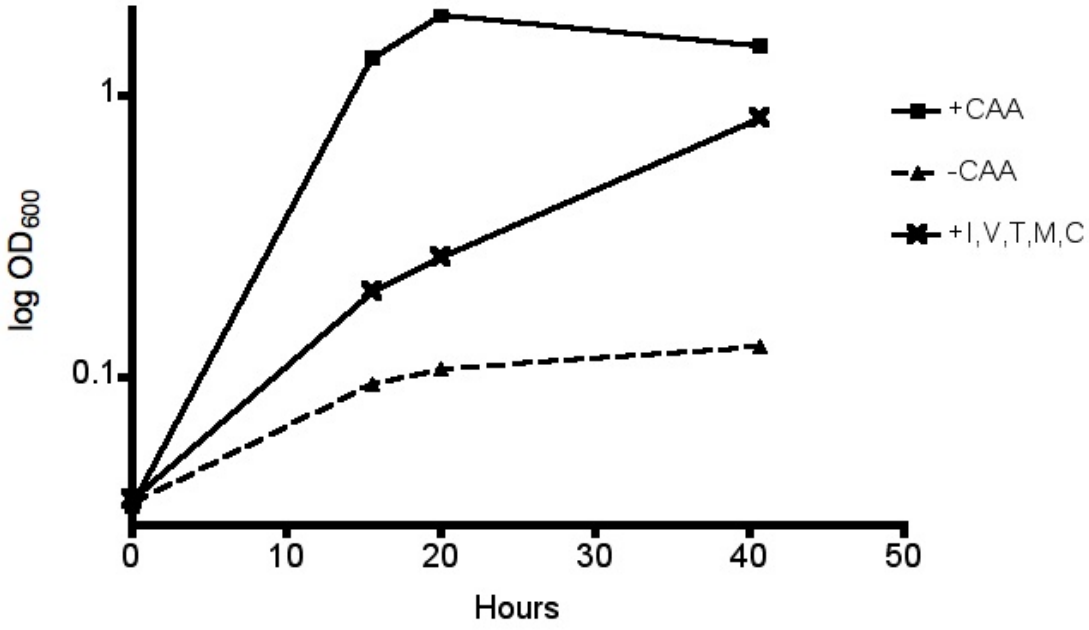


Figure 5. Growth of ADB267 in AMM with amino acid supplements. ADB267 was grown anaerobically for 2 days in AMM+0.1% Casamino acids (CAA). Culture was diluted 1:10 into fresh AMM+CAA and grown until mid-log phase, at which point it was sub-cultured 1:20 into fresh AMM with indicated supplements. Individual amino acids were added to 250µg/ml, except valine, which was added to 500µg/ml. Cultures were grown at 37°C with shaking at 250rpm in chamber to which oxygen had been added. Samples were taken over time and the OD_{600} was measured and recorded. The oxygen concentration was determined at the end of the experiment to be 0.8% using the pyrogallol method. CAA=Casamino acids. I=isoleucine. V=valine. T=threonine. M=methionine. C=cysteine.

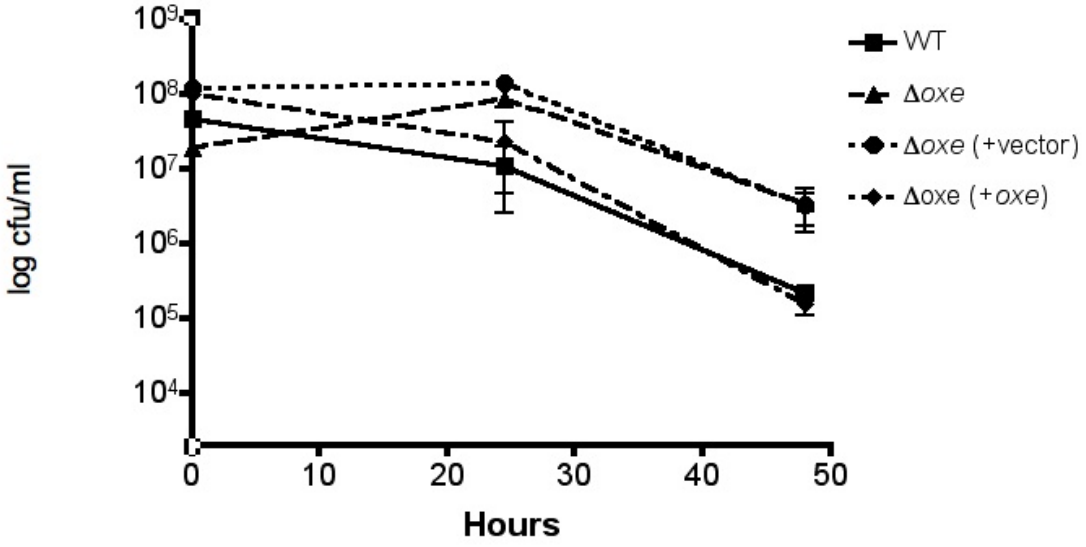


Figure 6. Δ*oxe* is more aerotolerant than wild-type. WT (ADB77), Δ*oxe* (ADB267), Δ*oxe* +vector (pJST61), and Δ*oxe* + *oxe*$^+$ (pADB293) cultures were grown anaerobically in BHIS to mid-log phase. Cells were pelleted and washed with PBS, and then resuspended in 10ml PBS+thymine+0.5% glucose in 125ml foam-stoppered flasks. Flasks were shaken at 37°C under room air. Samples were taken over time and serially diluted in PBS. 10µl aliquots were spotted to pre-reduced BHIS plates (±erm) and incubated in the anaerobic chamber. Colony-forming units were enumerated after 2 days incubation using a stage microscope.

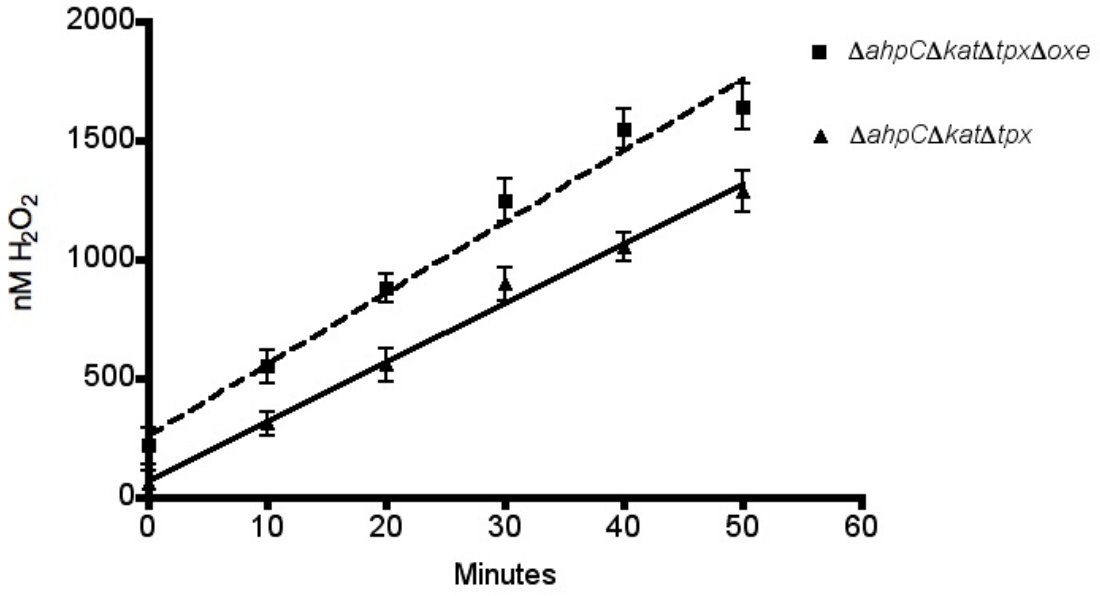


Figure 7. A Δ*oxe* strain produces ROS faster than wild-type. Strains BM105 (Δ*kat*Δ*ahpC*Δ*tpx*) and BM104 (Δ*kat*Δ*ahpC*Δ*tpx*Δ*oxe*) were grown to log-phase and then centrifuged. After resuspending pellets in AMM lacking cysteine, cultures were shaken for 1 hour at 37°C under room air. Cells were again centrifuged, washed, and resuspended in PBS+thymine to an OD_{600}=0.1. Freshly-prepared glucose was added to 0.05% just before start of assay. Suspensions were shaken at 37°C under room air throughout the course of the assay. Samples were taken over time and cells were centrifuged. Supernatants were assayed for [H_2O_2] using the Amplex Red protocol.

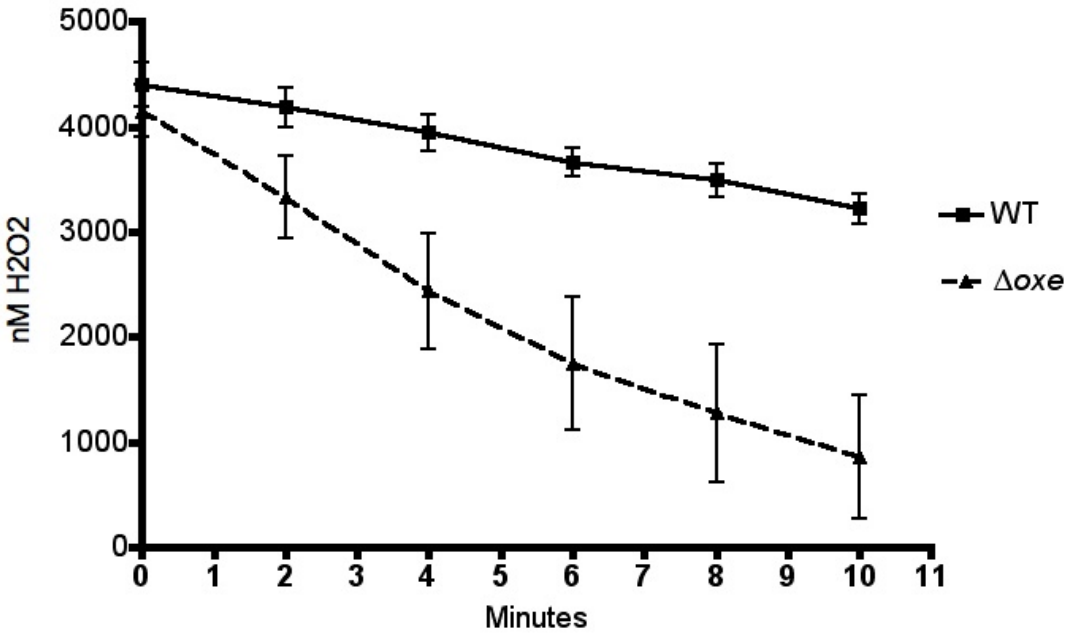


Figure 8. H_2O_2 scavenging rates of ADB77(squares) and ADB267(triangles). Mid-log phase cells were washed and resuspended in PBS+glucose to OD_{600}=0.3. H_2O_2 was added at time=0, at which point samples were taken. After centrifuging briefly, supernatants were assayed for [H_2O_2] using the Amplex Red reaction. Samples were taken over time to determine rates of peroxide disappearance.

References

1. **Babior, B. M., J. T. Curnutte, and R. S. Kipnes.** 1975. Biological defense mechanisms. Evidence for the participation of superoxide in bacterial killing by xanthine oxidase. J Lab Clin Med **85:**235-244.
2. **Baughn, A. D., and M. H. Malamy.** 2003. The essential role of fumarate reductase in haem-dependent growth stimulation of *Bacteroides fragilis*. Microbiology **149:**1551-1558.
3. **Baughn, A. D., and M. H. Malamy.** 2002. A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle. Proc Natl Acad Sci U S A **99:**4662-4667.
4. **Baughn, A. D., and M. H. Malamy.** 2004. The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. Nature **427:**441-444.
5. **Carlioz, A., and D. Touati.** 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J **5:**623-630.
6. **Farr, S. B., R. D'Ari, and D. Touati.** 1986. Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. Proc Natl Acad Sci U S A **83:**8268-8272.
7. **Fridovich, I.** 1998. Oxygen toxicity: a radical explanation. J Exp Biol **201:**1203-1209.

8. **Gardner, A. M., R. A. Helmick, and P. R. Gardner.** 2002. Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. J Biol Chem **277:**8172-8177.

9. **Godoy, V. G., M. M. Dallas, T. A. Russo, and M. H. Malamy.** 1993. A role for *Bacteroides fragilis* neuraminidase in bacterial growth in two model systems. Infect Immun **61:**4415-4426.

10. **Goodman, A. L., N. P. McNulty, Y. Zhao, D. Leip, R. D. Mitra, C. A. Lozupone, R. Knight, and J. I. Gordon.** 2009. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe **6:**279-289.

11. **Guiney, D. G., P. Hasegawa, and C. E. Davis.** 1984. Plasmid transfer from *Escherichia coli* to *Bacteroides fragilis*: differential expression of antibiotic resistance phenotypes. Proc Natl Acad Sci U S A **81:**7203-7206.

12. **Hanahan, D., J. Jessee, and F. R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol **204:**63-113.

13. **Henle, E. S., and S. Linn.** 1997. Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J Biol Chem **272:**19095-19098.

14. **Herren, C. D., E. R. Rocha, and C. J. Smith.** 2003. Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. Gene **316:**167-175.

15. **Hill, S., S. Viollet, A. T. Smith, and C. Anthony.** 1990. Roles for enteric d-type cytochrome oxidase in N_2 fixation and microaerobiosis. J Bacteriol **172:**2071-2078.

16. **Hillmann, F., R. J. Fischer, F. Saint-Prix, L. Girbal, and H. Bahl.** 2008. PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*. Mol Microbiol **68:**848-860.

17. **Hondorp, E. R., and R. G. Matthews.** 2004. Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. PLoS Biol **2:**e336.

18. **Imlay, J. A.** 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem **77:**755-776.

19. **Imlay, J. A.** 2006. Iron-sulphur clusters and the problem with oxygen. Mol Microbiol **59:**1073-1082.

20. **Imlay, J. A.** 2003. Pathways of oxidative damage. Annu Rev Microbiol **57:**395-418.

21. **Jang, S., and J. A. Imlay.** 2007. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J Biol Chem **282:**929-937.

22. **Keyer, K., and J. A. Imlay.** 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proc Natl Acad Sci U S A **93:**13635-13640.

23. **Kim, S. O., K. Merchant, R. Nudelman, W. F. Beyer, Jr., T. Keng, J. DeAngelo, A. Hausladen, and J. S. Stamler.** 2002. OxyR: a molecular code for redox-related signaling. Cell **109:**383-396.

24. **Korshunov, S., and J. A. Imlay.** 2010. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. Mol Microbiol **75:**1389-1401.

25. **Kuo, C. F., T. Mashino, and I. Fridovich.** 1987. alpha, beta-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. J Biol Chem **262:**4724-4727.

26. **Liochev, S. I., and I. Fridovich.** 1993. Modulation of the fumarases of *Escherichia coli* in response to oxidative stress. Arch Biochem Biophys **301:**379-384.

27. **Massey, V.** 1994. Activation of molecular oxygen by flavins and flavoproteins. J Biol Chem **269:**22459-22462.

28. **Massey, V., S. Strickland, S. G. Mayhew, L. G. Howell, P. C. Engel, R. G. Matthews, M. Schuman, and P. A. Sullivan.** 1969. The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochem Biophys Res Commun **36:**891-897.

29. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem **244:**6049-6055.

30. **McCord, J. M., B. B. Keele, Jr., and I. Fridovich.** 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. Proc Natl Acad Sci U S A **68:**1024-1027.

31. **Messner, K. R., and J. A. Imlay.** 2002. Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. J Biol Chem **277:**42563-42571.

32. **Mongkolsuk, S., and J. D. Helmann.** 2002. Regulation of inducible peroxide stress responses. Mol Microbiol **45:**9-15.

33. **Pan, N., and J. A. Imlay.** 2001. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. Mol Microbiol **39:**1562-1571.

34. **Privalle, C. T., and E. M. Gregory.** 1979. Superoxide dismutase and O2 lethality in *Bacteroides fragilis*. J Bacteriol **138:**139-145.

35. **Rocha, E. R., C. D. Herren, D. J. Smalley, and C. J. Smith.** 2003. The complex oxidative stress response of *Bacteroides fragilis*: the role of OxyR in control of gene expression. Anaerobe **9:**165-173.

36. **Rocha, E. R., G. Owens, Jr., and C. J. Smith.** 2000. The redox-sensitive transcriptional activator OxyR regulates the peroxide response regulon in the obligate anaerobe *Bacteroides fragilis*. J Bacteriol **182:**5059-5069.

37. **Rocha, E. R., T. Selby, J. P. Coleman, and C. J. Smith.** 1996. Oxidative stress response in an anaerobe, *Bacteroides fragilis*: a role for catalase in protection against hydrogen peroxide. J Bacteriol **178:**6895-6903.

38. **Rocha, E. R., and C. J. Smith.** 1998. Characterization of a peroxide-resistant mutant of the anaerobic bacterium *Bacteroides fragilis*. J Bacteriol **180:**5906-5912.

39. **Rocha, E. R., and C. J. Smith.** 1999. Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. J Bacteriol **181:**5701-5710.

40. **Rocha, E. R., A. O. Tzianabos, and C. J. Smith.** 2007. Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe *Bacteroides fragilis*. J Bacteriol **189:**8015-8023.

41. **Sund, C. J., W. Greg Wells, and C. Jeffrey Smith.** 2006. The *Bacteroides fragilis* P20 scavengase homolog is important in the oxidative stress response but is not controlled by OxyR. FEMS Microbiol Lett **261:**211-217.

42. **Sund, C. J., E. R. Rocha, A. O. Tzianabos, W. G. Wells, J. M. Gee, M. A. Reott, D. P. O'Rourke, and C. J. Smith.** 2008. The *Bacteroides fragilis* transcriptome response to oxygen and H_2O_2: the role of OxyR and its effect on survival and virulence. Mol Microbiol **67:**129-142.

43. **Tang, Y. P., M. M. Dallas, and M. H. Malamy.** 1999. Characterization of the Batl (Bacteroides aerotolerance) operon in *Bacteroides fragilis*: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in in vivo model systems. Mol Microbiol **32:**139-149.

44. **Thompson, J. S., and M. H. Malamy.** 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from *Bacteroides fragilis* TAL2480 reveals strong similarity between CfiA and *Bacillus cereus* beta-lactamase II. J Bacteriol **172:**2584-2593.

45. **Varel, V. H., and M. P. Bryant.** 1974. Nutritional features of *Bacteroides fragilis subsp. fragilis.* Appl Microbiol **28:**251-257.

46. **Varghese, S., Y. Tang, and J. A. Imlay.** 2003. Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. J Bacteriol **185:**221-230.

47. **Vita, N., E. C. Hatchikian, M. Nouailler, A. Dolla, and L. Pieulle.** 2008. Disulfide bond-dependent mechanism of protection against oxidative stress in

pyruvate-ferredoxin oxidoreductase of anaerobic *Desulfovibrio* bacteria. Biochemistry **47:**957-964.

48. **Wildschut, J. D., R. M. Lang, J. K. Voordouw, and G. Voordouw.** 2006. Rubredoxin:oxygen oxidoreductase enhances survival of *Desulfovibrio vulgaris hildenborough* under microaerophilic conditions. J Bacteriol **188:**6253-6260.

49. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res **17:**3469-3478.

50. **Woodmansee, A. N., and J. A. Imlay.** 2002. Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. J Biol Chem **277:**34055-34066.

Chapter 3

Assessment of the Microaerobic Growth Capacity of Clinical and Fecal Strains of *Bacteroides fragilis*

Abstract

Bacteroides fragilis can only grow in environments containing ≤0.05% oxygen, but we have shown that spontaneous mutants capable of growth in 1% O_2 can arise at a frequency of ~10^{-6} relative to anaerobic controls. All such mutants analyzed to date contain lesions in the *oxe* gene, and also show increased tolerance to room air. *In vivo*, *B. fragilis* resides in the anaerobic environment of the large intestine, but it is known to cause infections in oxygenated tissues. We therefore tested whether fecal strains or strains isolated from these oxygenated sites of infection could grow under increased O_2 concentrations, and if such strains had alterations in *oxe*.

All of the 14 fecally-derived strains tested showed efficiencies of plating <10^{-5} in the presence of 0.5-1% oxygen, while clinical strains isolated from sites of infection displayed a much larger range in plating efficiencies. Ten of the clinical isolates grew with greater than 10% efficiency under microaerobic conditions, while 14 gave rise to colonies with frequencies similar to the fecal strains. Seven had intermediate phenotypes, with plating efficiencies between 10^{-5}-10^{-2}.

The *oxe* locus was sequenced for 7 clinical strains that grew well microaerobically. The deduced protein sequence indicated that 4 of these isolates encoded a wild-type Oxe protein while the remaining 3 had amino acid substitutions. This indicates that *oxe* mutants may arise or be selected for during *B. fragilis* clinical infections.

Introduction

Bacteroides fragilis is a mammalian commensal that resides in the anaerobic environment of the large intestine. In this setting, the organism is well protected from the toxic effects of oxygen. However, when released from the intestines into oxygenated tissue, they are exposed to relatively high concentrations of O_2 and susceptible to oxidative damage. For instance, in the peritoneal cavity there is between 2% (4) and 7% oxygen (7) . *B. fragilis* appears to be remarkably resistant to these brief encounters with oxygenated environments, as it remains viable long enough for abscess formation. With the assistance of facultative anaerobes such as *E. coli*, these abscesses can be rendered anaerobic, thus allowing *B. fragilis* to proliferate outside of its intestinal niche. *B. fragilis* is particularly adept at the transition from intestine to abscess, for while it makes up only about 5% of all *Bacteroides* in the intestine, it is found in almost 80% of anaerobic infection (2). While certain strains produce lipases, proteases, heparinases, and nucleases (5) that likely aid in the establishment of these abscesses, the aerotolerance of this organism also plays a key role. We have recently shown (chapter 2) that mutants of *B. fragilis* can be isolated that grow in microaerobic conditions and tolerate room air better than the wild-type, and that these "enabled" strains all had mutations in a gene called *oxe*. Because strains of *B. fragilis* associated with clinical infections would have encountered oxygenated environments, we wondered if they would be similarly enabled relative to fecal isolates. We therefore tested the ability of clinical and fecal isolates to grow under microaerobic conditions, and sequenced the *oxe* gene from several of these strains to

determine if differences in oxygen tolerance could be correlated with changes in the sequence of this critical protein.

Materials and Methods

Growth conditions

Anaerobiosis was maintained by using a Coy anaerobic chamber (Coy Laboratory Supplies, Grass Lake, MI) containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Strains were grown in brain heart infusion broth supplemented with 0.5% yeast extract and 15µg/ml hematin (BHIS).

DNA sequencing

Oligonucleotide primers used in this study were synthesized by IDT (Iowa City, IA) and are listed in Table 1. *In silico* analysis of DNA and protein sequences was performed using DNA STRIDER 1.2 (DNAstar, Madison, WI). PCR amplification was done with HotStarTaq Master Mix (Invitrogen, Carlsbad, CA). DNA sequencing was performed with the corresponding primers by the Tufts University Nucleic Acids and Protein Core Facility.

adr2	GGGCCATGGGCCACATTCCCTCAAAGAGG
adr6	CCTGGGATCCGATCTGATACTTGAGCG
adr7	CGCCGGATCCGAAACGGACTGATAGTCCG
BAM32	GGACCATTCAGGTTCTATCC
BAM33	GGTGTGGTAATTGCATACGG
BAM34	CTCAGTCCATACCGGACCG

Table 1. List of primers used in this study

Bacterial strains

All *B. fragilis* strains used in this study are listed in Table 2.

Efficiency of plating

Single colonies were used to inoculate BHIS, and these cultures were grown overnight at 37°C in the anaerobic chamber. Aliquots of these dense cultures were diluted 1:20 into fresh BHIS and grown to mid-log phase. Cultures were serially diluted in the anaerobic chamber using growth medium or PBS, and 100µl aliquots were spread on BHIS plates that had been reduced in the anaerobic chamber overnight. Plates were transferred to a chamber containing 0.5-1% oxygen and incubated at 37°C for several days. 10µl aliquots of these dilutions were also spotted to BHIS plates and left to incubate in the anaerobic chamber. After several days, colony-forming units (cfus) were enumerated. Oxygen concentrations were determined using the pyrogallol method (1), or a Coy oxygen analyzer (model 630).

The efficiency of plating was calculated by dividing the number of colonies formed under microaerobic conditions by the number formed anaerobically.

Strain	**Origin**	**Source**
Bf1	Fecal	D. Hecht

Bf10	Fecal	D. Hecht
6-2	Fecal	TAL
6-3	Fecal	TAL
10-1	Fecal	TAL
11-3	Fecal	TAL
13-11	Fecal	TAL
13-3	Fecal	TAL
PA5	Fecal	L. Comstock
CL100	Fecal	L. Comstock
B110	Fecal	L. Comstock
B124	Fecal	L. Comstock
1279-2	Fecal	L. Comstock
1284-2	Fecal	L. Comstock
21616	Pelvic abscess	TAL
21618	Right leg wound	TAL
21619	Abdomen	TAL
21620	Peritoneal fluid	TAL
21621	?	TAL
21622	Buttocks	TAL
21624	Anorectal	TAL
21625	Left foot tissue	TAL
21626	Coccyx tissue	TAL

21627	Toe bone	TAL
21628	Peritoneal fluid	TAL
21632	Chest fluid	TAL
21633	Abdominal wall tissue	TAL
22499	Blood	TAL
06110	?	NEMC
91107	Blood	NEMC
22539	Blood	TAL
22453	Blood	TAL
14061	Blood	D. Hecht
13082	Blood	D. Hecht
14106	Blood	D. Hecht
11805	Blood	D. Hecht
11786	Blood	D. Hecht
11716	Blood	D. Hecht
11773	Blood	D. Hecht
11759	Blood	D. Hecht
13303	Blood	D. Hecht
13314	Blood	D. Hecht
13324	Blood	D. Hecht
13328	Blood	D. Hecht
13344	Blood	D. Hecht

22508	Appendix	TAL
22551	Abscess	TAL
22557	Peritoneal abscess	TAL
22507	Abscess	TAL
22549	knee	TAL
22500	Wound	TAL

Table 2. List of strains used in this study. All were confirmed as *B. fragilis* by their source laboratories. Origins are listed for each isolate if known. TAL=Tufts Anaerobic Laboratory (Boston, MA), NEMC (Tufts Medical Center, Boston, MA), L. Comstock (Channing Lab, Boston, MA), D. Hecht (Loyola University, Chicago, IL).

Results

All of the strains studied grew well under anaerobic conditions, and generally formed colonies faster than our standard strain (ADB77) when streaked on plates and grown anaerobically. As shown in figure 1, none of the 14 fecally-derived strains grew well under microaerobic conditions, plating with efficiencies of ~$10^{-5.5}$ or lower relative to the anaerobic controls. These efficiencies are very similar to that of ADB77, although the parent of this strain (TM4000) was originally isolated as a clinical strain.

Isolates derived from clinical infections showed a much wider range in plating efficiencies under microaerobic conditions, with one isolate (22453) showing no growth while several others grew with ≥100% efficiency when oxygen was present. The origin of the strains did not appear to influence plating efficiencies in any discernable way. Additionally, none of strains tested showed significant growth under 2% oxygen.

In addition to the efficiency of plating assay described above, a simple qualitative plate assay was used to assay the microaerobic growth capacity of some strains. Strains were streaked to BHIS or anaerobic blood agar plates (from Tufts Anaerobe Lab) and placed under 0.5-1% oxygen. Susceptible strains fail to show robust growth in the primary streak, while enabled strains (like ADB267) grow well throughout all three streaks. Strains showing some growth in the secondary streak were considered “slightly enabled.”

Of 18 strains tested in this assay, 3 were found to be enabled (22508, 22551, and 22557) and two were “slightly enabled” (22507 and 22549).

We have previously demonstrated that *Bacteroides* strains capable of growth under microaerobic conditions have mutations in *oxe* (Chapter 2). Therefore, we PCR amplified the *oxe* region from several of the clinical strains using primers adr6 and adr7, and sequenced the product with the primers listed above. 22539, 21616, 21620, and 21621 (plating efficiencies $\geq 10^{-3}$) all showed slight differences in *oxe* sequence relative to ADB77, but only one (21621) gave rise to an altered predicted protein sequence (L394F).

The *oxe* sequence was also determined for 22551 and 22557 (both “enabled”) as well as 22500 (“susceptible”). Polymorphisms in the DNA sequence indicated that 22551 and 22557 were probably siblings, but both showed predicted Oxe amino acid sequences that differed from ADB77 (P205L). The amino acid sequence of 22500 Oxe matched that of ADB77.

Discussion

Comparison of the microaerobic plating efficiencies of fecal and clinical isolates of *B. fragilis* revealed dramatic differences. All fecal strains gave rise to colonies at very low frequencies, similar to the wild-type lab strain. Based on the studies outlined in chapter 2 of this report, we would hypothesize that the few colonies that did grow under microaerobic conditions have spontaneous mutations in the *oxe* gene. Clinical strains, however, showed a range of plating efficiencies that covered almost 8 orders of magnitude. Strikingly, more than one-third of those strains assayed plated with 10% efficiency or higher in the presence of 0.5-1% oxygen. 14 of the 31 clinical isolates plated with efficiencies similar to fecal strains, while the remaining 7 had an intermediate phenotype. These results suggest that *B. fragilis* strains isolated from sites of clinical infection have a greater chance of being "oxygen-enabled" relative to those derived from their intestinal niche. However, caution is warranted in analyzing these results, as these strains were received from different sources, and we cannot rule out the possibility that differences in handling and preserving of the bacteria affected their plating efficiencies. Additionally, patient-matched clinical/fecal samples would allow for more informative comparisons, as differences in oxygen tolerance would be less likely to be the result of strain variations.

Sequence analysis of *oxe* alleles yielded intriguing results. Overall, sequenced strains shared greater than 99% identity at the DNA level. This was a surprising finding given

that these strains had originated from very different sources. A remarkable conservation of sequence was seen at the amino acid level as well, with 5 of the sequenced strains matching ADB77 exactly. Three showed changes in the predicted Oxe protein sequences, and 2 or those appeared to be siblings. This indicates that it may be possible to isolate clinical strains with mutations in *oxe*.

We do not currently know the role of Oxe in the anaerobic physiology of *B. fragilis*. Studies with transposon-insertion mutant pools of *B. thetaiotaomicron* suggest that Oxe is essential for proliferation *in vivo* (3) . However, these were most likely null mutants, whereas amino acid substitutions like those seen in the clinical strains described here might preserve Oxe's anaerobic function while giving rise to an oxygen-enabled strain. It therefore remains possible that mutations in the *oxe* gene could give rise to oxygen-enabled strains *in vivo*, although our results clearly show that there are alternative pathways to growing microaerobically. Perhaps most interesting are those strains plating with intermediate efficiencies of between 10^{-5}-10^{-2}. This could be the result of stoichiometric differences in the level of some "O_2-tolerance switch" within populations of these strains. For instance, if Oxe is in fact preventing microaerobic growth of these strains, then some fraction of cells might show reduced transcription of the gene. In this case, oxygen adaptation would not be the result of mutation, but of regulatory control.

While we cannot make definitive statements regarding the oxygen tolerance of clinical and fecal strains of *Bacteroides*, the results described here highlight some important

differences between the two. A more exhaustive survey with additional samples may be warranted.

Acknowledgments

We are grateful to members of the Tufts Anaerobe Laboratory and Tufts Medical Center, as well as to Laurie Comstock and David Hecht for the gift of strains.

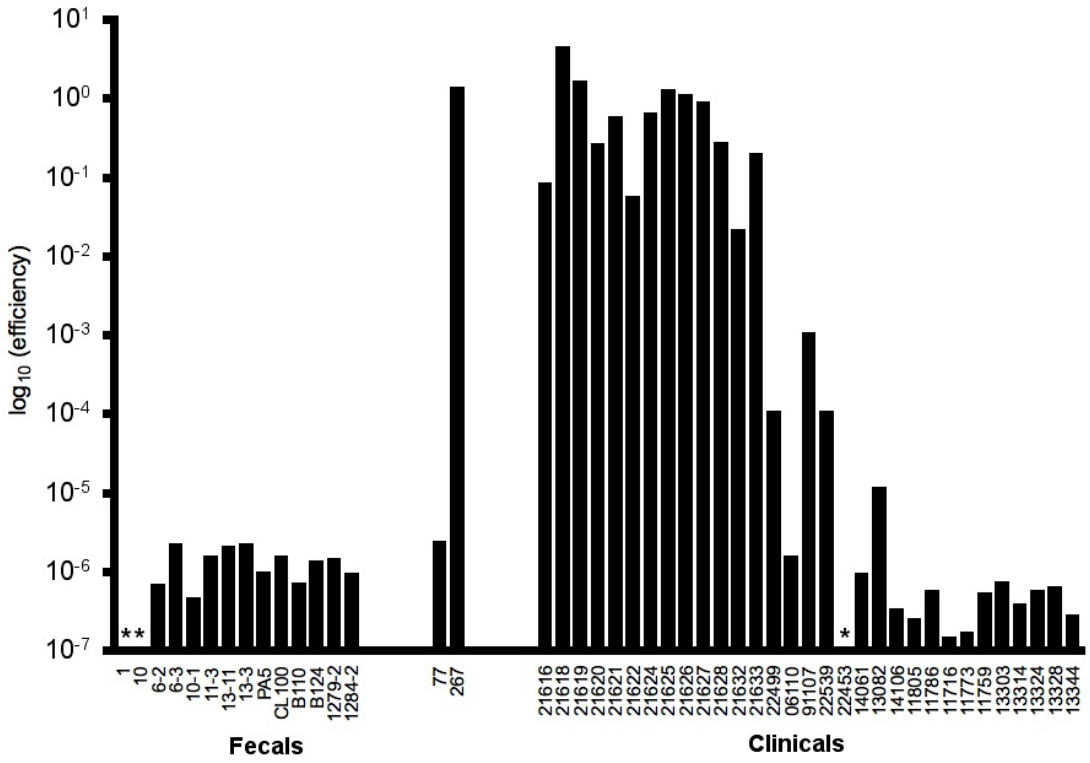


Figure 1. Efficiencies of plating under microaerobic conditions for various clinical and fecal strains of *B. fragilis*. Cultures were grown under anaerobic conditions at 37°C to mid-log phase, then serially diluted. 100µl aliquots were spread to pre-reduced BHIS plates and transferred to chamber containing 0.5-1% oxygen. Plates were incubated at 37°C for 3-5 days. Alternatively, 10µl aliquots of dilutions were spotted to pre-reduced BHIS and left to incubate anaerobically. Colony-forming units were enumerated, and efficiencies of plating were calculated as indicated above. ADB77(wild-type) and ADB267 (ADB77Δ*oxe*) are shown as references. *=below the limit of detection.

References

1. **Baughn, A. D., and M. H. Malamy.** 2004. The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. Nature **427:**441-444.
2. **Brook, I.** 1989. Anaerobic bacterial bacteremia: 12-year experience in two military hospitals. J Infect Dis **160:**1071-1075.
3. **Goodman, A. L., N. P. McNulty, Y. Zhao, D. Leip, R. D. Mitra, C. A. Lozupone, R. Knight, and J. I. Gordon.** 2009. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe **6:**279-289.
4. **Leavitt, M.** 1970. Oxygen Tension in the Gut. New England Journal of Medicine **282:**1039-1040.
5. **McGowan, K., and S. L. Gorbach.** 1981. Anaerobes in mixed infections. J Infect Dis **144:**181-186.
6. **Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett.** 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. J Infect Dis **136:**82-89.
7. **Sawyer, R. G., M. D. Spengler, R. B. Adams, and T. L. Pruett.** 1991. The peritoneal environment during infection. The effect of monomicrobial and polymicrobial bacteria on pO_2 and pH. Ann Surg **213:**253-260.

Chapter 4

Identification of Genes Involved in Growth of *Bacteroides fragilis* on Amino Sugars

Abstract

Though *Bacteroides fragilis* can grow on amino sugars as sole carbon sources, many of the enzymes necessary for this process have not been identified. In *E. coli* NagA (N-acetylglucosamine deacetylase) and NagB (glucosamine 6-P deaminase) play key roles in amino sugar utilization. Sequences that show some similarities to both enzymes have been identified in *B. fragilis*. In this report we show that neither of the two potential NagA homologues appear to be involved in catabolism of the amino sugar N-acetylglucosamine (NAG). A potential NagB1 homologue can function in the same capacity as the *E. coli* NagA, most likely via a predicted deacetylase domain. Deletion of the *nagB1* gene prevented growth of *B. fragilis* on NAG, but did not affect growth on glucose or glucosamine. Additionally, a vector-borne copy of *B. fragilis* NagB1 complemented an *E. coli* Δ*nagA* mutant for growth on NAG, but did not allow growth of an *E. coli* Δ*nagB* strain on the same carbon source. Another NagB homologue in *B. fragilis*, NagB2, rescued growth of the *E. coli* Δ*nagB* strain on NAG and glucosamine when present *in trans*, indicating that it can function as a glucosamine 6-P deaminase. Delineating the role of NagB2 in *B. fragilis* metabolism was complicated by an inability to delete the gene, but a mutant strain carrying a carboxy-terminal insertion in this gene (*nag*B2*) could be isolated. This mutant demonstrated wild-type growth on all sugars tested, but a Δ*oxe*/*nagB2** double mutant failed to grow on NAG and glucosamine, and could only grow on glucose if 10% peptone, tryptone, or supplemented brain-heart infusion broth were added to the minimal medium. It is therefore possible that Oxe and NagB2 participate in amino sugar utilization in *B. fragilis*.

Introduction

The role of Oxe in preventing growth of *B. fragilis* under microaerobic conditions has been extensively detailed in chapter 2. However, the role of this enzyme, if any, in anaerobic growth is unknown. Directly downstream of *oxe* is a gene encoding a putative glucosamine 6-P deaminase (NagB), and its close proximity suggests that these two genes may form an operon. NagB plays a key role in the catabolism of amino sugars like glucosamine (GlcN) and N-acetylglucosamine (NAG) in *E. coli* (fig. 1), but the protein has not yet been studied in *Bacteroides*. Because co-regulated genes often encode enzymes in a related pathway, we sought to test whether Oxe and NagB are involved in the utilization of these amino sugars in *B. fragilis*.

As an intestinal commensal, *B. fragilis* has evolved to utilize complex polysaccharides and host-derived glycoproteins as efficient carbon sources (10). These glycoproteins provide an abundant source of the amino sugars such as NAG. The kinases necessary for activation of glucosamine and NAG have been identified (5), but the specific enzymes necessary for funneling these phosphorylated amino sugars into glycolysis have not be characterized. With this work we hope to gain insights into these pathways and perhaps connect them to the oxygen-enabled phenotype seen in *oxe* mutants.

Materials and Methods

Reagents

IPTG and N-acetylglucosamine were purchased from Sigma (St. Louis, MO) and glucosamine was a generous gift of Ted Park. All other chemicals were from Fisher Scientific.

Growth conditions

Anaerobiosis was maintained by using a Coy anaerobic chamber containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. *B. fragilis* was grown in brain heart infusion broth supplemented with 0.5% yeast extract and 15µg/ml hematin (BHIS) or anaerobic minimal medium (AMM) containing 0.5% of the appropriate carbon source, as previously described (4). In some cases super anaerobic minimal medium (SAMM) plates were used (12). Because all strains are *thyA* mutants, thymine was added to 50µg/ml. Gentamicin (50µg/ml), rifampicin (50µg/ml), trimethoprim (80µg/ml) and tetracycline (2.5µg/ml) were added to plates where appropriate. *E. coli* used for matings was grown aerobically in L broth, and chloramphenicol (25µg/ml), ampicillin (100µg/ml), kanamycin (25µg/ml), and tetracycline (10µg/ml) were added where appropriate. For complementation experiments, *E. coli* MG1665 and derivatives were grown aerobically at 37°C in M63+0.5µg/ml vitamin B1 containing 0.5% of the appropriate carbon source.

Strains and Plasmids

Strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α was used for cloning, and strain HB101/pRK231 was used for mobilization of plasmids from DH5α to *B. fragilis* recipient strains. DH5α was made competent for transformation through use of the RbCl method previously described (9) .

Strain	Relevant characteristics	Source
B. fragilis		
ADB77	TM4000Δ*thyA*, Tp^R	(4)
ADB267	ADB77Δ*oxe*	(chapter 2)
BM62	ADB77 *nagB2**	This work
BM63	ADB267 *nagB2**	This work
CJB Δ*nagB1*	ADB77 Δ*nagB1*	Lab strain
BM64	CJB Δ*nagB1*/*nagB2**	This work
BM76	ADB77 Δ*nagA1*	This work
BM75	ADB77 Δ*nagA2*	This work
BM81	BM75 Δ*nagA1*	This work
E. coli		
DH5α	λ nonlysogen	(13)

HB101	*rpsL20*, host strain for pRK231	(11)
MG1655	F^- λ^- *ilvG rfb50 rph1*	Lab stock
MG1655 Δ*nagA*		This work
MG1655 Δ*nagB*		This work
Plasmids		
pADB242	pYT102 derivative, 0·35 kb *Bam*HI–*Hin*dIII fragment replaced by 18 bp *Bam*HI–*Hin*dIII fragment from pCR2.1-TOPO (Invitrogen) Cm^R	(3)
pRK231	RP4 derivative, Tet^R, Tra^+	(7)
pADB242::Δ*nagA1*	Contains deletion construct for *B. fragilis nagA1*	This work
pADB242::Δ*nagA2*	Contains deletion construct for *B. fragilis nagA1*	This work
pADB242::*nagB2**	Contains disruption for *B. fragilis nagB2*	This work
pTrc99A	pBR322*ori*, $lacI^q$, *trc* promoter, *rrnB* terminator, Amp^R	Pharmacia Biotech (Stockholm, Sweden)

pTrc99A::*nagB2*	*B. fragilis nagB2* ligated to promoter via NcoI site	This work
pTrc99A::*nagB1*	*B. fragilis nagB1* ligated to promoter via NcoI site	This work
pTrc99A::*nagA1*	*B. fragilis nagA1* ligated to promoter via NcoI site	This work
pTrc99A::*nagA2*	*B. fragilis nagA2* ligated to promoter via NcoI site	This work

Table 1. Strains and plasmids used in this work.

DNA manipulation

Primers used in this study are listed in Table 2. Primer sequences were designed using to the *B. fragilis* NCTC9343 annotated sequence found on the Pedant3 webpage (http://pedant.gsf.de) and synthesized by IDT (Iowa City, IA). Genomic DNA was amplified using HotStarTaq Master Mix (Invitrogen, Carlsbad, CA). Plasmid and PCR product purifications were performed with QIAprep spin columns (Qiagen, Chatsworth, CA). Where indicated, DNA was digested with restriction enzymes purchased from New England Biolabs (Beverly, MA). Ligations were performed using T4 DNA Ligase from Invitrogen.

Primer	Region of homology	Sequence
61RAB	pADB242	GGCGCGCCGTAAGGAAAGTGGCTCTCAG
1843	pADB242	CCCATCGGTGATGTCGGC
BAM33	*oxe*	GGTGTGGTAATTGCATACGG
adr6	Upstream of *oxe*	CCTGGGATCCGATCTGATACTTGAGCG
BAM129	*nagB2*	ACATGGATCCTGCTGCTAAGATCAAAGCTG
BAM130	*nagB2*	ACATGGATCCGGTGATCTGCTTCGATATCCT
BAM131	*nagB1*	TCCCATCATTTATCGTTCCC
BAM132	*nagB1*	GCTTCGGCGGTATCGATA
BAM135	*nagB1*	TTTCAAGGTCTACGGCAG
BAM136	*nagB1*	CATTCGCAGGAAGTGACC
BAM139	*nagB2*	ATCACCATGGGGATTGGCAGCTTTGATCTT
BAM140	*nagB2*	ACATCCATGGGTTTGCGATGATGCTGCTAC
BAM144	*nagB2*	ATTGGTCTCACATGAGACTAATCATTCAGCC
BAM145	*nagB2*	CATACTGCAGTATCGGAGGGATTGAACCTT
BAM146	*nagB1*	ATTGGTCTCACATGAAGACAAATCTTAGTTC
BAM147	*nagB1*	TCATGGATCCGAGTTCCAAACTCCTCACTC
BAM176	Upstream of *nagA1*	ACATGGATCCTATTAGCCGAACCGG
BAM177	*nagA1*	CATATCTAGAGTCTTTCATCCATCCTTG
BAM178	*nagA1*	CTACTCTAGAGCCGATATTCTGGTACTG

BAM179	Downstream of *nagA1*	TACTGCGGCCGCAGTATTCAAAGATGG
BAM180	Upstream of *nagA1*	CAACGGAGATTGTTGTTTTACCG
BAM181	Upstream of *nagA2*	CATAGGATCCGTCTACCTGGTCGATATG
BAM182	*nagA2*	ACCTTCTAGAACCTTCGTTCAACC
BAM183	*nagA2*	TCACTCTAGACCAGAAGGATAAAGATGC
BAM184	Downstream of *nagA2*	CATAGCGGCCGCTTTTAAACCAAGCCC
BAM185	Upstream of *nagA2*	CTCAGGTTCTTGATTTGCGCATAG

Table 2. Primers used in this study.

Strain and plasmid construction

All *B. fragilis* deletion mutants were constructed using a double-crossover technique previously described (8). In order to build a deletion construct for *nagA1*, a fragment consisting of 754bp of *nagA1* upstream sequence and 57bp of *nagA1* amino-terminal coding sequence was amplified using primers BAM176 and BAM177. The downstream fragment was made by PCR amplifying a region consisting of 90bp of *nagA1* carboxy-terminal coding sequence and 741bp of downstream sequence with primers BAM178 and BAM179. After purifying PCR products, the upstream fragment was digested with

BamHI and XbaI, while the downstream fragment was digested with XbaI and NotI. These digested products were purified and ligated via a three-way reaction to pADB242 that had been digested with BamHI and NotI to create pADB242::Δ*nagA1*.

The suicide plasmid pADB242Δ*nagA1* was delivered to the recipient *B. fragilis* strain as previously described (11). Tetracycline-resistant colonies were screened for the appropriate cointegrant event using primers 1843 and BAM179 as well as 61RAB and BAM176. Isolates demonstrating recombination at the *nagA1* locus were grown overnight in BHIS with thymine to allow for recombination events leading to the resolution of the disrupting plasmid. The presence of the *thyA* gene on pADB242 sensitizes cointegrants to trimethoprim, while those resolvants that have excised the plasmid are trimethoprim-resistant. For this reason, the culture was plated to SAMM containing glucose, thymine, and trimethoprim. Colonies arising after 3-4 days were purified and then screened for tetracycline-sensitivity on BHIS+thymine plates. Tet^S colonies were then used as template in a PCR with primers BAM 179 and BAM180 to identify Δ*nagA1* clones.

The deletion construct for *nagA2* was created by PCR amplifying a region consisting of 717bp of *nagA2* upstream sequence and 63bp of amino-terminal coding sequence with primers BAM181 and BAM182. A fragment containing 94bp of carboxy-terminal coding sequence and 700bp of downstream sequence was amplified with primers BAM183 and BAM184. Again the upstream sequence was digested with BamHI and XbaI while the downstream fragment was digested with XbaI and NotI. These were

ligated to the BamHI/NotI-digested pADB242 to create pADB242::Δ*nagA2*. The procedure described above was used to create cointegrants, which were screened by PCR with primers 1843 and BAM184 as well as 61RAB and BAM181. Resolvants were screened with primers BAM184 and BAM185.

The disruption construct for *nagB2* was made by first PCR amplifying 728bp of *nagB2* coding sequence using primers BAM129 and BAM130. After purifying PCR fragment, it was digested with BamHI and ligated to pADB242 that had also been digested with BamHI to give pADB242::*nagB2**. The procedure outline above was used to create cointegrants, and these were screened for insertions at *nagB2* using primers BAM33 and 1843 for strains containing the wild-type *oxe* gene, and adr6 and 1843 for Δ*oxe* strains.

The *nagB2* complementing construct was made by first PCR amplifying the *nagB2* gene with primers BAM144 and BAM145. This product was purified and digested with PstI and BsaI. Plasmid pTrc99A was digested with PstI and NcoI and ligated to the *nagB2* fragment. With this method, the start codon for *nagB2* was directly fused to the *trc* promoter to make pTrc99A::*nagB2*. The construct was confirmed to be correct by sequencing with primers BAM139, BAM140, BAM144, BAM145.

The *nagB1* complementing construct was made by PCR amplifying the *nagB1* gene using primers BAM146 and BAM147. The purified product was digested with BamHI and BsaI and ligated to pTrc99A that had been digested with BamHI and NcoI. The construct

was confirmed to be correct with primers BAM131, BAM132, BAM135, and BAM136 as well as pTrc99A-specific primers.

To create Δ*nagA* and Δ*nagB* derivatives of MG1655, we first obtained strains from the Keio collection with deletions in those genes (2). These strains were grown overnight in L broth with kanamycin. Calcium chloride was added to the culture to 5mM. 100µl of phage P1 stock was added and 1ml was transferred to glass tube. The tube was incubated in a 37°C water bath for 20 minutes and 2.5 ml supplemented agar (0.8% agar + 2mM $CaCl_2$ + 0.1% glucose) was then added. Solution was mixed briefly and poured onto a fresh L broth plate. The plate was incubated for 5 hours at 37°C. Soft agar was scraped into a glass test tube and, after washing the surface of the plate was with 1ml L broth, this was also added to the tube. Several drops of chloroform were added, the solution was vortexed for 30 seconds, and then centrifuged at 4°C. The supernatant was transferred to a fresh glass tube, several drops of chloroform were added, and the solution was vortexed. Phage stock was titered on MG1655 and found to contain ~10^{11} plaque-forming units/ml. To transduce cultures, MG1655 was first grown overnight in L broth at 37°C. 1ml was centrifuged and resuspended in 1ml MC buffer (100mM $MgSO_4$ + 5mM $CaCl_2$). 400µl of cell suspension was added to two 1.5ml microcentrifuge tubes. 100µl P1 lysate was added to one tube, and 100µl of 1:10 dilution of P1 lysate was added to the other. The tubes were incubated in 37°C water bath for 12 minutes, then 400µl 0.1M sodium citrate was added. After vortexing for 30 seconds, cell suspension was combined with 4ml L broth + 0.1M sodium citrate in a glass test tube. Culture was incubated at 30°C for one hour. 2ml of culture was then centrifuged and pellet was resuspended in

200µl 0.1M sodium citrate. This was spread on L plate containing kanamycin and 0.1M sodium citrate and incubated overnight at 37°C. Plates contained dozens of colonies, while neither P1 lysate alone nor MG1655 cells alone gave rise to colonies on these plates. After purifying colonies to L+kan+citrate plates, 2 were grown overnight in 5ml L+kan. Electrocompetent cells were prepared from these overnight cultures, and each strain was electroporated with pCP20 (6). Cells were allowed to recover for 1 hour at 37°C, then plated to L broth plates containing chloramphenicol. Overnight incubation yielded dozens of colonies, which were purified to the same medium. One colony was used to inoculate 5ml L broth and tube was incubated overnight at 42°C without shaking. A loopful of this culture was streaked to an L plate. After overnight incubation at 37°C, isolated colonies were patched to L, L+chloramphenicol, and L+kanamycin. After confirming that all colonies were sensitive to both antibiotics, one was grown overnight at 37°C and saved as MG1655Δ*nagA* or MG1655Δ*nagB*.

Results

B. fragilis encodes multiple homologues of NagA and NagB

BLAST analysis of the *B. fragilis* genome using *E. coli* NagA and NagB as queries resulted in the identification of two potential NagAs and two potential NagBs. NagA1 and NagA2 share 62.7% and 63.3% total homology to the *E. coli* protein respectively, and share 80.7% homology between one another. NagB1, a protein predicted to contain 664 amino acids, shared 25.9% homology over the first half of the sequence with the *E. coli* NagB, which contains 266 residues. Within the carboxy terminus of NagB1 is a potential *pigL* domain, predicted to encode a N-acetylglucosaminyl phosphatidylinositol de-N-acetylase. NagB2 shares 78.2% total homology with the *E. coli* NagB. The genomic arrangement of these loci is depicted in figure 2.

Oxe may play a role in the utilization of N-acetylglucosamine and glucosamine

Multiple attempts at deleting *nagB2* in *B. fragilis* were unsuccessful. However, an insertion near the carboxy-terminus of this gene allowed us to test the role of NagB2 in the utilization of NAG and GlcN as sole carbon sources. As seen in figures 3 and 4, a strain bearing a *nagB2* disruption (*nagB2**) could still grow on glucose, NAG, or GlcN. Likewise, a Δ*oxe* strain grew well on all of these carbon sources. However, a Δ*oxe*/*nagB2** double mutant could not grow on either glucosamine or NAG (fig. 3B and

4A). Additionally, when this strain was diluted 1:10 into AMM+glucose from BHIS, it grew like wild-type for several hours, but then began to show signs of lysis. Samples viewed under a microscope showed large amounts of cell debris and very few whole cells, indicating that the strain had a major growth defect in glucose. When BHIS-grown cultures of Δ*oxe*/*nagB2** were diluted 1:100 into AMM+glucose, they showed no signs of growth (data not shown), unless the medium was supplemented with 10% tryptone or peptone. In this case, the culture grew as if it had been supplemented 1:10 from BHIS. 0.25% Casamino acids did not allow for growth under these conditions.

NagB1 can function as an N-acetylglucosamine 6-P deacetylase but not a glucosamine 6-P deaminase

A *B. fragilis* Δ*nagB1* strain grew well in AMM supplemented with glucose or glucosamine (data not shown and fig. 4A). However, it was unable to grow when NAG was supplied as the only carbon source. This was also the case for a Δ*nagB1*/*nagB2** strain (fig. 4B). According to the NAG utilization pathway of *E. coli* (fig. 1), this would seem to indicate that NagB1 was not a NagB, but rather had characteristics more similar to NagA. To test this, *nagB1* was cloned into a vector that placed it under the control of an IPTG-inducible promoter. This plasmid was introduced into *E. coli* strains missing *nagA* and *nagB*. As seen in figure 5A, the *B. fragilis nagB1* gene could complement an *E. coli* Δ*nagA* strain for growth on NAG when no IPTG was added to the culture. IPTG was found to inhibit growth in a concentration-dependent manner from 0.01-1mM.

nagB1 could not, however, complement a Δ*nagB* strain of *E. coli* either with or without IPTG (fig. 5B).

NagB2 can function as a glucosamine 6-P deaminase

As seen in figure 5B, when *B. fragilis nagB2* was cloned into a vector under the control of an IPTG-inducible promoter, it was able to complement an *E. coli* Δ*nagB* mutant for growth on NAG. This was also the case for growth on glucosamine (data not shown). Again, IPTG inhibited growth of this strain in a concentration-dependent manner from 0.01-1mM.

Neither NagA1 nor NagA2 appear to function as N-acetylglucosamine 6-P deacetylases

Deletions of *nagA1* and *nagA2* from *B. fragilis* appeared to have no effect on the ability of the organism to grow on NAG, even when deleted in tandem (fig. 6). Additionally, their presence in a Δ*nagB1* strain is not enough to allow growth on NAG (fig. 6), indicating that while they bear homology to the *E. coli* N-acetylglucosamine 6-P deacetylase, they do not appear to encode such a function.

Discussion

The proximity of a gene encoding a putative glucosamine 6-P deaminase to the *oxe* locus on the *B. fragilis* genome led us to investigate the potential role of Oxe in the utilization of NAG and GlcN as carbon sources. While a Δ*oxe* strain showed no growth defect on these substrates, the introduction of a mutant *nagB2* allele (*nagB2**) into this background not only prevented growth on NAG and GlcN, but severely impaired the organism's ability to utilize glucose. The functionality of this altered NagB2* is unclear, as there was no phenotype associated with a strain carrying this allele in an otherwise wild-type background. NagB2* is predicted to have an amino acid sequence exactly matching the wild-type allele over the first 262 residues. However, the carboxy-terminus of NagB2* contains an extra 37 amino acids, thereby creating a protein of 307 residues. The lack of growth of a Δ*oxe*/*nagB2** on NAG and GlcN is confounding, and is made more so by the altered growth on glucose, as alterations in pathways for amino sugar utilization should not directly impact growth on glucose. The ability of this double mutant to grow in AMM+glucose when supplemented with 10% BHIS, tryptone, or peptone, however, is intriguing. These supplements do not appear to be satisfying an amino acid auxotrophy, as Casamino acids could not rescue growth. It is possible that they stimulate growth by acting as osmoprotectants, as work in *Listeria monocytogenes* has demonstrated that this organism cannot grow in a high-salt medium without peptone supplementation (1), but can grow well in BHI. If this is the case with *B. fragilis*, it implies that the Δ*oxe*/*nagB2** is experiencing high-salt stress from some intracellular or extracellular source.

113 bp upstream of the likely translational start site of *oxe* is a putative -10 *B. fragilis* binding sequence (TATTTTTG) for RNA polymerase, although there is no obvious -35 sequence. The predicted translational start site for *nagB2* lies 40 bp downstream of the *oxe* coding sequence, indicating that these two genes may be co-regulated. However, there are potential -10 (TAAATTTG) and -35 (TTTG) sequences in the carboxy-terminal coding sequence of *oxe* (starting at nucleotide 1080 and 1066, respectively) that may play a role in transcription of *nagB2*. The strains described as Δ*oxe* in this study are missing this potential -10 sequence, so the inability of the Δ*oxe*/*nagB2** strain to grow on various sugars may relate to a misregulation of *nagB2** expression.

Clearly *B. fragilis* differs from *E. coli* in the pathways for amino sugar utilization. While *E. coli* employs NagA for deacetylation of N-acetylglucosamine 6-P, the two NagA homologues in *B. fragilis* do not appear to serve this purpose. Instead, a protein bearing some homology to NagB (NagB1) can function in this capacity, most likely due to its *pigL*-like deacetylase domain. Recent work has also shown that while wild-type *B. fragilis* can grow on N-acetylgalactosamine as a sole carbon source, a Δ*nagB1* strain cannot use this amino sugar (M. Malamy, personal communication), indicating this enzyme can most likely deacetylate multiple substrates. It is unclear what role(s) are served by NagA1 and NagA2.

NagB2 appears to function as a glucosamine 6-P deaminase, as the *B. fragilis* homologue was able to rescue growth of an *E. coli* Δ*nagB* mutant on NAG and GlcN. The sensitivity

of the complemented *E. coli* strains to IPTG-mediated overexpression of both *nagB2* and *nagB1* most likely indicates that this organism must tightly control transcription of these genes. However, the role of this enzyme in *B. fragilis* physiology could not be exhaustively tested due to an inability to delete the gene. This could indicate that NagB2 is essential for *in vitro* growth, although it is not clear why this would be.

Overall, the results in this report have led to a more complete understanding of the amino sugar utilization pathways employed by *B. fragilis*, and these findings are reflected in the schematic shown in figure 7. Further work is needed to identify other proteins involved in this process, and may help to explain the ability of this commensal to maintain its foothold in its intestinal niche.

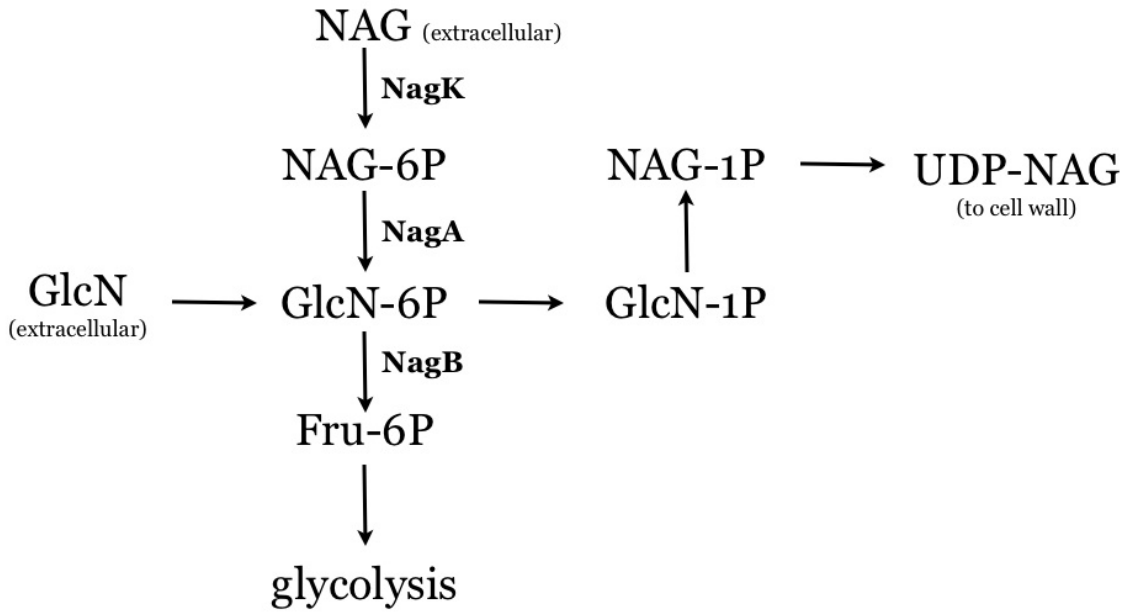


Figure 1. Pathway for utilization of N-acetylglucosamine and glucosamine in *E. coli*

NAG=N-acetylglucosamine, GlcN=glucosamine, Fru=fructose, NagK=NAG kinase, NagA=Nag-6P deacetylase, NagB=Glcn-6P deaminase, UDP=uridine diphosphate.

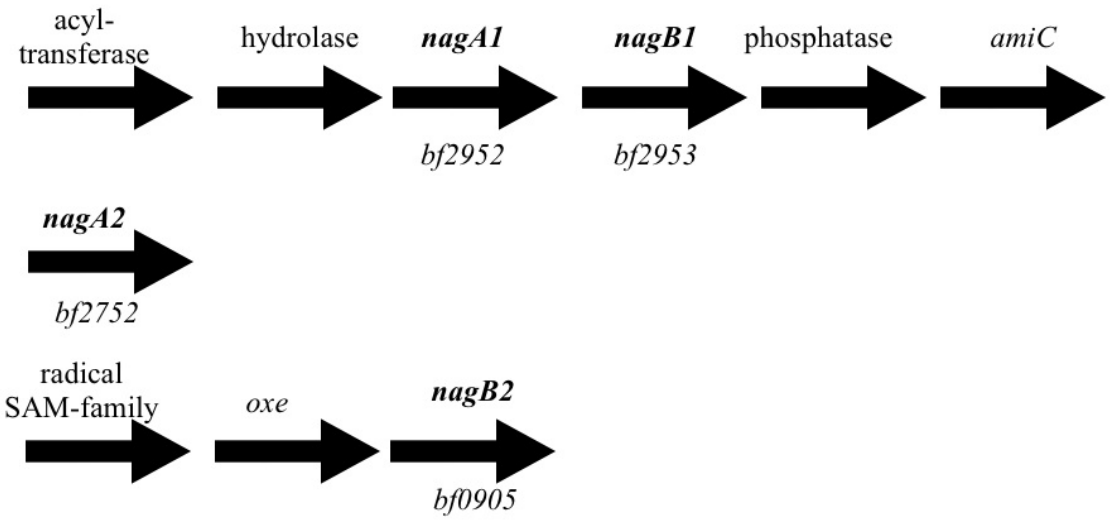
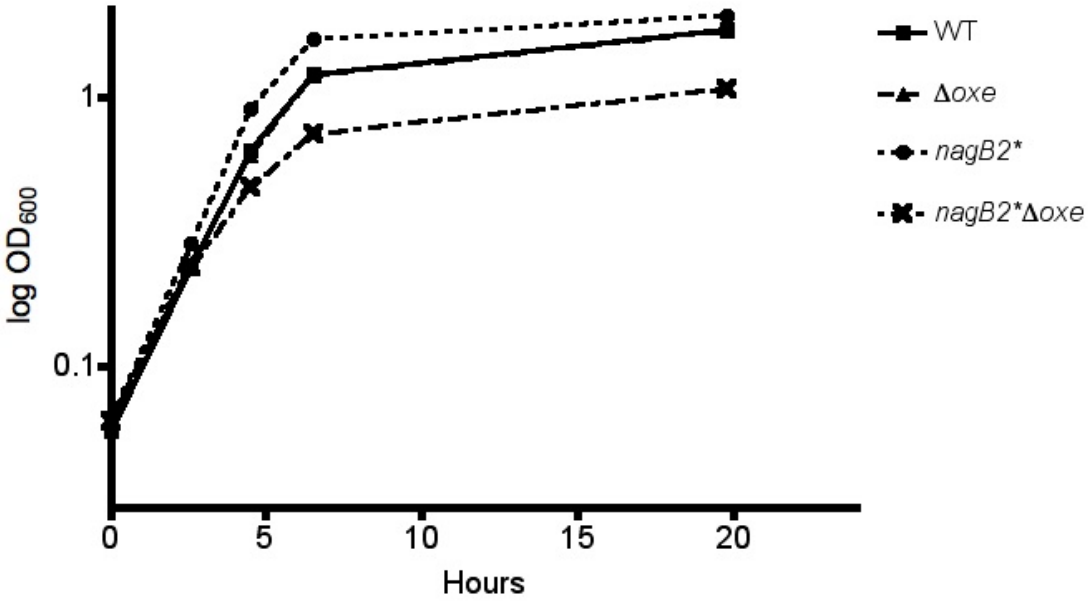


Figure 2. Genomic map showing *B. fragilis* homologues of *E. coli nagA* and *nagB* genes. Gene numbers are shown below arrows. *amiC*=N-acetylmuramoyl-L-alanine amidase.

A.


log OD600
1
0.1
0
5
10
15
20
Hours
WT
Δoxe
nagB2*
nagB2*Δoxe

B.

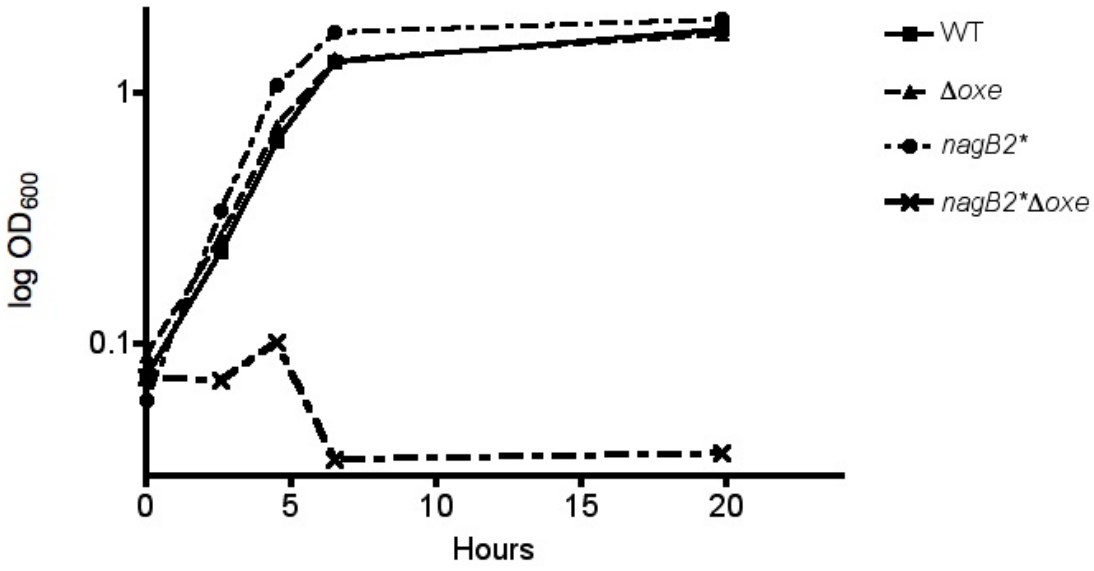
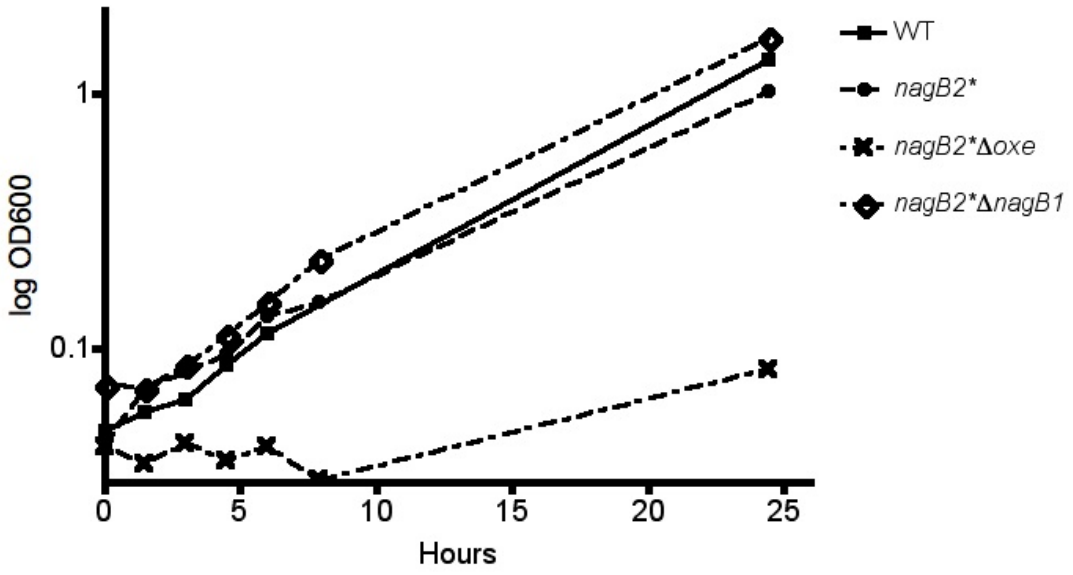

log OD600
1
0.1
0
5
10
15
20
Hours
WT
Δoxe
nagB2*
nagB2*Δoxe

Figure 3. Growth of ADB77 (WT), ADB267 (Δ*oxe*), BM62 (*nagB2**), and BM63 (*nagB2**Δ*oxe*) in AMM + 0.5% glucose (A) and 0.5% N-acetylglucosamine (B). Strains were grown overnight at 37°C in BHIS (±tetracycline). Cultures were diluted ~1:100 and grown to mid-log phase in same medium. These cultures were diluted to OD_{600}~0.05 in AMM containing the indicated sugar and shaken at 37°C. OD_{600} was monitored over time.

A.



B.

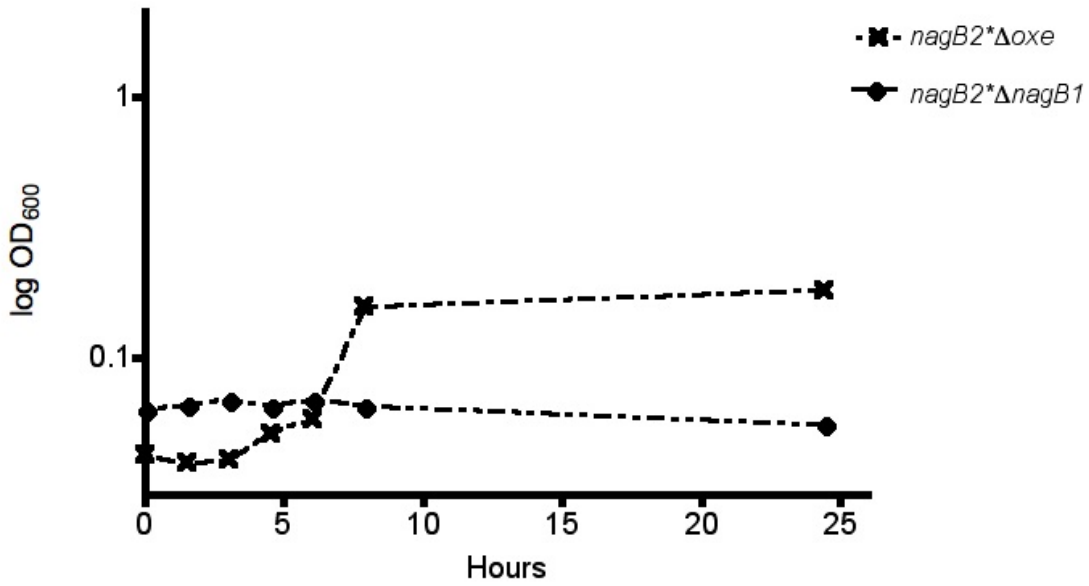
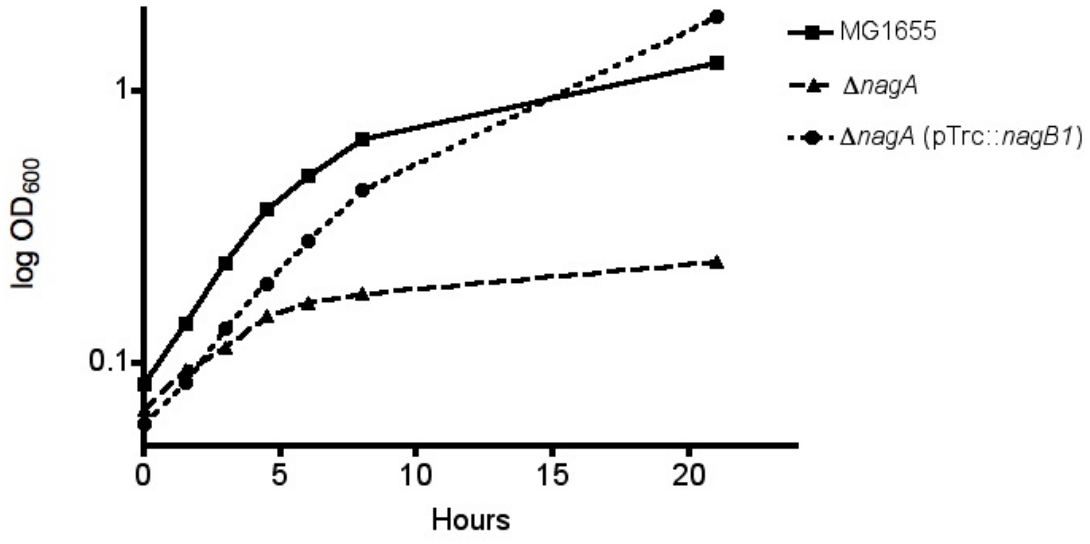


Figure 4. Growth of ADB77 (WT), ADB267 (Δ*oxe*), BM62 (*nagB2**), BM63

(*nagB2**Δ*oxe*) and BM64 (*nagB2**Δ*nagB1*) in AMM+0.5% glucosamine (A) or 0.5%N-acetylglucosamine (B). Strains were grown overnight in BHIS±tetracycline. These cultures were subbed ~1:200 into fresh medium and grown to mid-log phase. Cultures were centrifuged, and pellets were washed in anaerobic chamber with AMM. After centrifuging again, cells were resuspended in AMM and added to AMM+appropriate sugar at a starting OD_{600}~0.05. Cultures were shaken at 100rpm in the anaerobic chamber, and OD_{600} was monitored over time.

A.


log OD600
1
0.1
0
5
10
15
20
Hours
MG1655
ΔnagA
ΔnagA (pTrc::nagB1)

B.

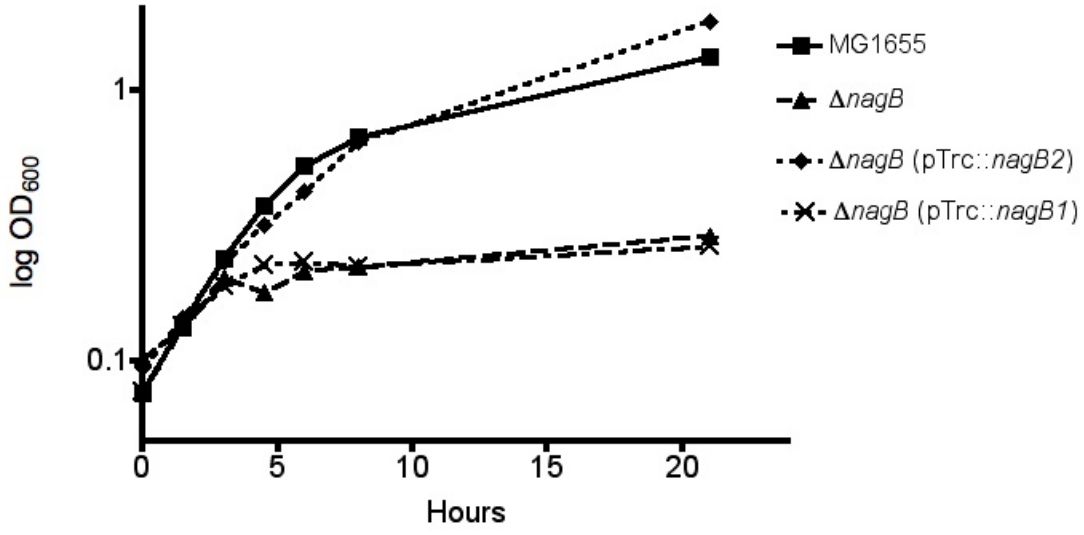

log OD600
1
0.1
0
5
10
15
20
Hours
MG1655
ΔnagB
ΔnagB (pTrc::nagB2)
ΔnagB (pTrc::nagB1)

Figure 5. Complementation of *E. coli* Δ*nagA* (A) and Δ*nagB* (B) strains with *B. fragilis nagB1* and *nagB2*. MG1655, MG1655Δ*nagA*, MG1655Δ*nagA* (pTrc99A::*B.fragilis nagB1*), MG1655Δ*nagB*, MG1655Δ*nagB* (pTrc99a::*B.fragilis nagB1*), and MG1655Δ*nagB* (pTrc99A::*B.fragilis nagB2*) were grown overnight in M63+vitamin B1+0.5% glucose (±ampicillin) at 37°C. Diluted to OD_{600}~0.1 in M63+B1 containing 0.5% NAG. Grew aerobically at 37°C, and followed OD_{600} over time.

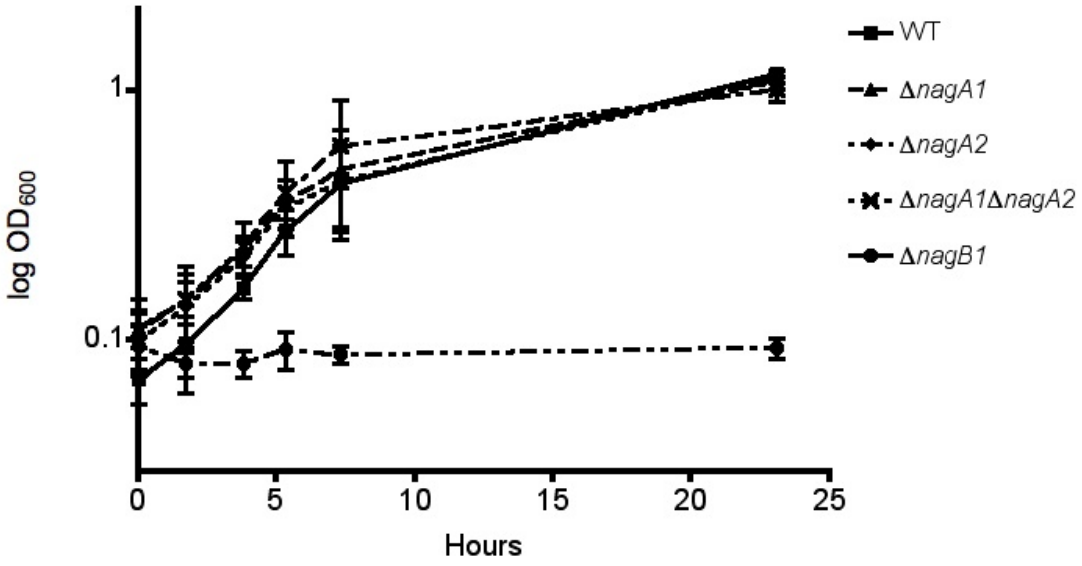


Figure 6. Growth of ADB77 (WT), BM76 (Δ*nagA1*), BM75 (Δ*nagA2*), BM81 (Δ*nagA1*Δ*nagA2*), and CJBΔ*nagB1* in AMM+0.5% N-acetylglucosamine. Strains were grown anaerobically overnight at 37°C in AMM+glucose. These cultures were diluted ~1:100 into fresh AMM+glucose and grown to mid-log phase. 1ml aliquots were removed, centrifuged for one minute anaerobically, and supernatants were removed. Pellets were resuspended in 10ml of AMM+0.5% NAG. OD_{600} was monitored over time.

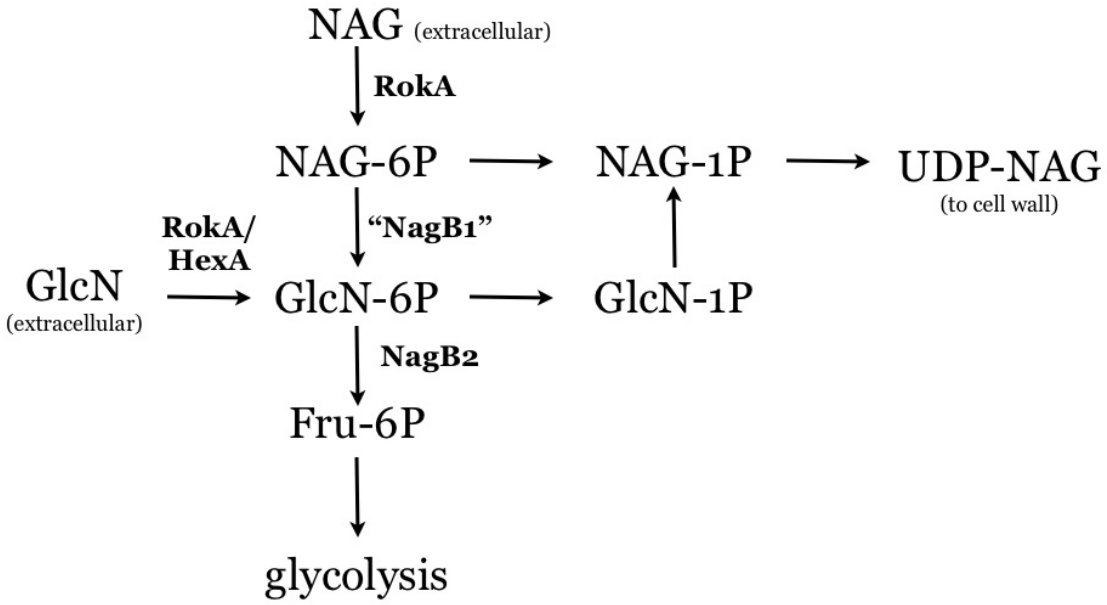


Figure 7. Predicted pathway for N-acetylglucosamine and glucosamine utilization in *B. fragilis*. Abbreviations are as in figure 1. RokA=Repressor orf kinase. Hex=hexose.

Acknowledgments

We thank Veronica Godoy for providing strains from Keio collection.

References

1. **Amezaga, M. R., I. Davidson, D. McLaggan, A. Verheul, T. Abee, and I. R. Booth.** 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. Microbiology **141 (Pt 1):**41-49.
2. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006 0008.
3. **Baughn, A. D., and M. H. Malamy.** 2003. The essential role of fumarate reductase in haem-dependent growth stimulation of *Bacteroides fragilis*. Microbiology **149:**1551-1558.
4. **Baughn, A. D., and M. H. Malamy.** 2002. A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle. Proc Natl Acad Sci U S A **99:**4662-4667.
5. **Brigham, C. J., and M. H. Malamy.** 2005. Characterization of the RokA and HexA broad-substrate-specificity hexokinases from *Bacteroides fragilis* and their role in hexose and N-acetylglucosamine utilization. J Bacteriol **187:**890-901.
6. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-6645.

7. **Godoy, V. G., M. M. Dallas, T. A. Russo, and M. H. Malamy.** 1993. A role for *Bacteroides fragilis* neuraminidase in bacterial growth in two model systems. Infect Immun **61:**4415-4426.

8. **Guiney, D. G., P. Hasegawa, and C. E. Davis.** 1984. Plasmid transfer from Escherichia coli to *Bacteroides fragilis*: differential expression of antibiotic resistance phenotypes. Proc Natl Acad Sci U S A **81:**7203-7206.

9. **Hanahan, D., J. Jessee, and F. R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol **204:**63-113.

10. **Hooper, L. V., T. Midtvedt, and J. I. Gordon.** 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr **22:**283-307.

11. **Thompson, J. S., and M. H. Malamy.** 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from *Bacteroides fragilis* TAL2480 reveals strong similarity between CfiA and *Bacillus cereus* beta-lactamase II. J Bacteriol **172:**2584-2593.

12. **Varel, V. H., and M. P. Bryant.** 1974. Nutritional features of *Bacteroides fragilis subsp. fragilis.* Appl Microbiol **28:**251-257.

13. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res **17:**3469-3478.

Conclusions and future directions

Oxidative stress is part and parcel of life on an oxygenated planet. While oxygen's strong affinity for electrons makes it an important part of the metabolic circuitry used by many organisms, it is also the feature that can cripple and destroy those same organisms. The adventitious transfer of electrons from cellular components to oxygen results in the generation of reactive oxygen species (ROS) that can wreak havoc on DNA and crucial biochemical pathways. In humans, ROS have been implicated in diseases like cancer, Alzheimer's disease, stroke, Parkinson's, multiple sclerosis, cystic fibrosis, and diabetes, to name just a few (1). Understanding the nature of these diseases requires knowledge of the mechanisms of ROS generation and detoxification. Bacteria provide an excellent model for investigating these processes due to their genetic tractability and the ease with which oxidative damage can be monitored. This is especially true for anaerobic bacteria, as identification of the vulnerabilities that preclude growth in aerobic environments can lead to important insights regarding oxygen toxicity.

Sequestered within the mammalian large intestine, *Bacteroides fragilis* should be safe from O_2 poisoning, yet it is known to encode a robust and nuanced response to oxygen exposure. In an environment that rarely sees oxygen, superoxide dismutase, catalase, and peroxidases would seem to be superfluous, yet the presence of genes encoding these enzymes in the *B. fragilis* genome would suggest that this organism comes into contact with O_2 on a somewhat regular basis. We have demonstrated that this oxidative stress response is multi-layered and somewhat redundant, as the effect of Tpx was seen only

when AhpC was missing. Why should an anaerobe encode six potential peroxidases? It is interesting to note that these predicted peroxidases could potentially reduce H_2O_2 at the expense of different electron donors. For AhpC, the ultimate reductant is NADH, for Tpx it is NADPH, and the other four are predicted to utilize glutathione (Glp), an unspecified thiol (Tps) and NAD(P)H (Ccp, Dyp). This would imply that *B. fragilis* stands ready to reduce H_2O_2 at all times, as at least one of those reductants should be available no matter the metabolic state of the cell. While we know that glutathione is not present, there is most likely another thiol-containing compound in this organism. Rocha and Smith suggested that thioredoxins may be the only compounds necessary for maintaining redox balance, yet the phenotypes associated with a thioredoxin reductase (TrxB) mutant appeared relatively minor (3). It is not difficult to imagine that a microbe with multiple peroxidases would also produce the electron carriers necessary to fuel them.

The overlapping layers of the oxidative stress response also highlight an important consideration in dealing with the cell's complex electronic circuitry. By deleting a redox-associated protein, the electrons that would normally flow to that enzyme are diverted elsewhere. This could be a major reason that deletions of individual peroxidases yield such unimpressive phenotypes, as removal of one enzyme simply increases the pool of reductant that can now be used by other peroxidases. It would be interesting to measure the redox state of *B. fragilis* when some of these enzymes are deleted. This can be done using a luminescence assay employing the *Vibrio harveyii* luciferase (6). Additionally, we have shown that a Δ*ahpC*Δ*kat*Δ*tpx* strain retains ~20% of the peroxide scavenging

activity of the wild-type. Additional deletions of *dyp*, *glp*, and *tps* did not appear to lower this activity significantly, but *ccp* has not been mutated in this background yet. Perhaps such a strain would be completely incapacitated in H_2O_2 detoxification.

The creation of the Δ*ahpC*Δ*kat*Δ*tpx* strain has been an important step in identifying endogenous ROS sources in *B. fragilis*. Deletions of other potential redox enzymes from this genetic background could yield information about other ROS generators. We have shown that fumarate reductase produces ~47% of the H_2O_2 in this organism when cells are exposed to room air, but this ratio could change under different oxygen concentrations. For instance, when *B. fragilis* is shaken under 21% O_2, fumarate levels likely drop precipitously due to the destruction of fumarase. Therefore, fumarate cannot compete with oxygen for the electrons carried by fumarate reductase, and ROS generation is maximal. However, exposure to 1% oxygen should allow some fumarase activity, so the elevated fumarate levels may severely reduce Frd-mediated ROS production. This can be easily tested by performing the Amplex Red protocol on cells shaken microaerobically.

Oxygen-enabled mutants that arise spontaneously are easily detected when *B. fragilis* is plated under microaerobic conditions, and so far all of these enabling mutations have mapped to *oxe*. However, the Δ*ahpC*Δ*kat*Δ*tpx*Δ*oxe* strain cannot grow under these conditions. Therefore, plating the Δ*ahpC*Δ*kat*Δ*tpx* mutant should not give rise to *oxe* mutants. However, we have recently found that this strain does give rise to enabled mutants at a frequency of ~10^{-6}. This might indicate that oxygen-enabled colonies can

arise through mutation of a different gene. If sequencing of the *oxe* locus indicates that it is intact, then creating a transposon-insertion library with the triple mutant may help identify other genes involved in the O_2-enabling phenomenon.

We have shown that Oxe plays a major role in sensitizing *B. fragilis* to oxygen, yet we do not know its role in anaerobic metabolism. Experiments testing its involvement in amino sugar utilization have yielded perplexing results, complicated by the fact that we were unable to delete the *nagB* homologue directly downstream of *oxe*. The inability of the Δ*oxe*/*nagB2** mutant to grow on various sugars indicates that these two gene products may participate directly or indirectly in amino sugar catabolism. It is interesting to note that a portion of the β-lactamase-like domain of Oxe bears some homology to *gloB*, a glyoxalase important for the detoxification of methylglyoxal (MG). This compound is synthesized by bacteria facing imbalances in intracellular concentrations of glucose 6-P or fructose 6-P due to mutation or misregulation of sugar utilization pathways (5). Production of MG via MG synthase (MgsA) from dihydroxyacetone phosphate is thought to relieve cells from the stress caused by the elevated levels of these phosphorylated sugars (2, 4), but accumulation of MG can also lead to cell death if it is not detoxified. The major pathway for detoxification involves first activating MG with glutathione. This produces a hemiacetal that acts as a substrate for GloA, which isomerizes it to S-lactoylglutathione. GloB can remove the glutathione from this intermediate, thus producing lactate. The production and detoxification of MG are illustrated in figure 1.

B. fragilis encodes homologues of MgsA (*bf3624*) and GloA (*bf3127*), suggesting that the methyglyoxal pathway is intact in *B. fragilis* if Oxe can act as a GloB. Due to the lack of glutathione, however, MG would have to be activated by a different thiol-containing compound, and the GloB-mediated reaction does not have a redox component to it making it less likely that Oxe could serve in this capacity. However, preliminary results showed that a Δ*oxe* strain was killed at a faster rate than wild-type when exposed to 2mM MG in minimal medium as assayed by viable counts. We were not able to detect any GloB-like enzymatic activity in wild-type extracts. If Oxe is indeed involved in MG detoxification, there could be an explanation for the lack of growth of the Δ*oxe*/*nagB2** strain on amino sugars. The NagB2* protein is not very different from the predicted wild-type NagB2, with the exception of a 37 amino acid C-terminal extension. If this protein is somehow more stable than the wild-type enzyme, it's possible that NagB2* is actually *more* active as a glucosamine 6-P deaminase rather than less. This would lead to an increased concentration of fructose 6-P when cells are grown on NAG or glucosamine, which could activate the production of MG. In such a scenario, a *nagB2** strain would grow perfectly well on amino sugars because it can detoxify MG, but deleting *oxe* from this strain would render it susceptible. It is also possible that Oxe is necessary not for the GloB-like reaction, but to reduce the thiol-containing compound needed for activation of MG. Purification and characterization of the NagB2* protein may help shed some light on these potential pathways, as would deletion of *mgsA* and *gloA*.

Overall, further characterization of Oxe's function should reveal important aspects of *B. fragilis*'s physiology. Combined with the identification of oxygen-enabling mutations in other organisms, we may truly begin to understand what makes an anaerobe an anaerobe.

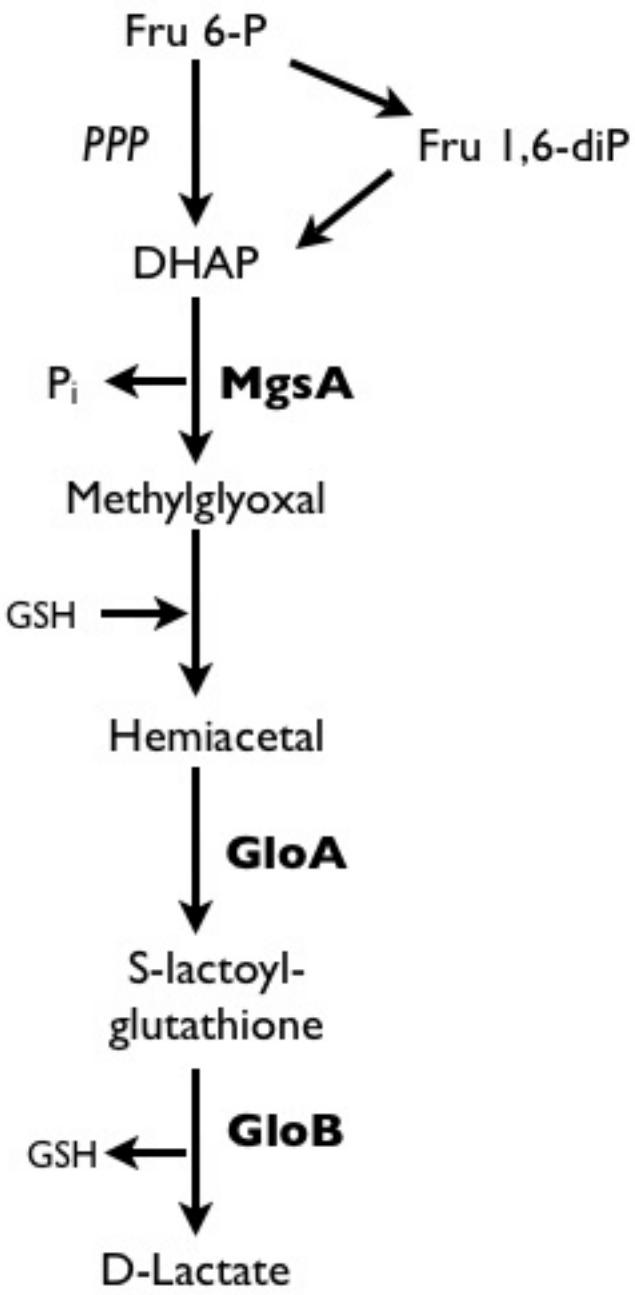


Figure 1. The pathway for production and glyoxalase-mediated detoxification of methylglyoxal. Fru=fructose, PPP=pentose phosphate pathway, DHAP=dihydroxyacetone phosphate, GSH=glutathione, P_i=inorganic phosphate.

References

1. **Halliwell, B., Gutteridge, J.** 1999. Free Radicals in Biology and Medicine, Third ed. Oxford University Press, Oxford, UK.
2. **Kadner, R. J., G. P. Murphy, and C. M. Stephens.** 1992. Two mechanisms for growth inhibition by elevated transport of sugar phosphates in Escherichia coli. J Gen Microbiol **138:**2007-2014.
3. **Rocha, E. R., A. O. Tzianabos, and C. J. Smith.** 2007. Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe Bacteroides fragilis. J Bacteriol **189:**8015-8023.
4. **Totemeyer, S., N. A. Booth, W. W. Nichols, B. Dunbar, and I. R. Booth.** 1998. From famine to feast: the role of methylglyoxal production in Escherichia coli. Mol Microbiol **27:**553-562.
5. **Weber, J., A. Kayser, and U. Rinas.** 2005. Metabolic flux analysis of Escherichia coli in glucose-limited continuous culture. II. Dynamic response to famine and feast, activation of the methylglyoxal pathway and oscillatory behaviour. Microbiology **151:**707-716.
6. **Woodmansee, A. N., and J. A. Imlay.** 2002. Reduced flavins promote oxidative DNA damage in non-respiring Escherichia coli by delivering electrons to intracellular free iron. J Biol Chem **277:**34055-34066.