Inactivation of a single gene enables microaerobic growth of the obligate anaerobe *Bacteroides fragilis*

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Bacteroides fragilis **can replicate in atmospheres containing ≤0.05% oxygen, but higher concentrations arrest growth by an unknown mechanism. Here we show that inactivation of a single gene, *oxe* (i.e., oxygen enabled) in *B. fragilis* allows for growth in concentrations as high as 2% oxygen while increasing the tolerance of this organism to room air. Known components of the oxidative stress response including the *ahpC*, *kat*, *batA-E*, and *tpx* genes were not individually important for microaerobic growth. However, a Δ*oxe* strain scavenged H_2O_2 at a faster rate than WT, indicating that reactive oxygen species may play a critical role in limiting growth of this organism to low-oxygen environments. Clinical isolates of *B. fragilis* displayed a greater capacity for growth under microaerobic conditions than fecal isolates, with some encoding polymorphisms in *oxe*. Additionally, isolation of oxygen-enabled mutants of *Bacteroides thetaiotaomicron* suggests that Oxe may mediate growth arrest of other anaerobes in oxygenated environments.**

superoxide | peroxidase | anaerobiosis

When cyanobacteria developed the ability to strip electrons from water ~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 (i.e., O_2) as a byproduct of photosynthesis left many microbes vulnerable to oxidative damage, caused in part by the reactivity of key components of the metabolic networks used by these organisms. Free iron and flavin-containing proteins (1–5) can adventitiously donate electrons to oxygen to produce reactive oxygen species (ROS) like superoxide (i.e., O_2^-) or hydrogen peroxide. These endogenously generated ROSs can destroy important iron–sulfur clusters, inactivate key metabolic enzymes, and cause DNA lesions that may culminate in death of the organism (6–13). 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 more robust ROS detoxifying enzymes like superoxide dismutase (14), rubrerythrins, catalases, and peroxidases (15), as well as metabolic enzymes fortified against ROS damage (16–18). 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.

Obligately anaerobic bacteria cannot replicate in the presence of oxygen, and present-day species may have evolved from the sediment-dwelling ancient microbes. For this reason, obligate anaerobiosis was once thought to be rooted in the lack of ROS-detoxifying enzymes (19). Extensive evidence to the contrary has since negated this hypothesis, but the molecular mechanisms underlying anaerobiosis have remained elusive. Although the possibility remains that molecular oxygen itself can cause growth cessation in this class of organisms (20), a complete picture of O_2 sensitivity has not yet taken shape.

Bacteroides fragilis provides a convenient 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 (21). In addition, even though it cannot replicate in room air, this organism is capable of mounting a strong response to aeration (22, 23), including the production of numerous ROS-scavenging enzymes (24–29) and other factors (30) that contribute to its impressive aerotolerance. The unique position of *B. fragilis* 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, as a result of mutation of a single gene (*oxe*), are capable of growth in as much as 2% oxygen, representing a 40-fold increase vs. WT *B. fragilis*. Additionally, an *oxe* mutant is even more tolerant of room air than its WT counterpart, thus providing an intriguing link between those bacteria that thrive in aerobic environments and those that do not.

Results

Isolation of Oxygen-Enabled Mutants. Recent work has shown that *B. fragilis* can grow under atmospheres containing as much as 0.05% oxygen (500 nM dissolved O_2) (21). Higher concentrations of O_2, however, inhibit growth in a manner independent of increases in environmental redox potential (31). With these observations in mind, we sought to isolate mutants of *B. fragilis* capable of growth under normally restrictive oxygen concentrations. When WT *B. fragilis* was plated under microaerobic conditions (0.25–2% oxygen by volume), colonies were found to form at a frequency of $\sim 10^{-6}$ relative to an anaerobic control (Table 1). Purification of these colonies revealed that they were capable of robust growth under microaerobic conditions, suggesting that a spontaneous mutation had somehow given rise to "oxygen-enabled" variants of *B. fragilis*. To gain insights into the molecular nature of this microaerobic growth, we tested whether various mutations on the *B. fragilis* genome would alter the frequencies at which oxygen-enabled colonies arose.

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 some of these proteins is activated at the transcriptional level by OxyR (23). A strain encoding a constitutively active allele of *oxyR* (32), as well as a Δ*oxyR* strain, were found to give rise to colonies at the same frequency as WT under microaerobic conditions (Table 1), indicating that the OxyR-mediated oxidative stress response (OSR) is neither sufficient nor essential for the oxygen-enabled phenotype. However, we reasoned that we might still be able to

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Table 1. Log_{10} efficiencies of plating for various strains of *B. fragilis* under microaerobic conditions

Strain	Relevant characteristics	Log_{10} (efficiency of plating)
ADB77	TM4000Δ*thyA*	−5.9
ADB267	ADB77Δ*oxe*	−0.03
ADB266	ADB77Δ*cydAB*	−6.3
BM37	ADB77Δ*kat*	−5.1
BM28	ADB77Δ*ahpC*	−5.0
IB263	638R*oxyR*^{C}	−6.3
MBD616	TM4000 *thyA*$_2$Δ*sodA*	−6.2
YT135	TM4000 *batD*::Tn*4400′*	−5.9
BM50	ADB77Δ*ahpC*Δ*kat*	−5.7
BM118	ADB77Δ*oxyR*	−5.9
BM95	ADB77Δ*tpx*	−4.9
BM131	ADB77 Δ*ahpC*Δ*tpx*	−5.2
BM105	ADB77Δ*ahpC*Δ*kat*Δ*tpx*	≤8.7
BM134	ADB77Δ*rubrerythrins*	−5.4
BM2	ADB77Δ*rub*	−4.9

Strains were grown anaerobically, serially diluted, and plated on BHIS. Test plates were transferred to microaerobic conditions, and control plates were transferred to anaerobic conditions. After several days, counts (in cfu) were enumerated. Log_{10} (efficiency of plating) is defined as the log_{10} (number of colonies arising in the presence of oxygen/number of colonies arising anaerobically).

suppress the formation of oxygen-enabled colonies by deleting enzymes crucial to the reduction of oxygen or its reactive intermediates. As cytochrome oxidase (*cydAB*) is used during nanaerobic growth of *B. fragilis* and appears to be the major reducer of O_2 under low-oxygen conditions (21), we hypothesized that it would also play a crucial role in microaerobic growth. However, a Δ*cydAB* strain plated with the same low efficiency as WT under microaerobic conditions, as did strains missing catalase (*kat*), superoxide dismutase (*sod*), alkylhydroperoxide reductase (*ahpC*), thioredoxin-dependent peroxidase (*tpx*), or the *Bacteroides* aerotolerance BAT operon (30) (Table 1), suggesting that a fully functional OSR was not necessary for the oxygen-enabled phenotype. Only when *ahpC*, *kat*, and *tpx* were deleted in combination were we unable to detect any oxygen-enabled colonies (Table 1).

One mutant, however, gave a surprising result. The *BF638R_0963* gene (National Center for Biotechnology Information Gene ID 11704906) encodes a predicted flavoprotein with homology to the nitric oxide reductase NorV, as well as to rubredoxin:oxygen oxidoreductase (Roo), an enzyme found to protect *Desulfovibrio vulgaris* Hildenborough under 1% oxygen by reducing O_2 to water (33). Based on this homology, we reasoned that deletion of BF638R_0963 would sensitize the organism to oxygen. Contrary to this prediction, we found that deletion of this gene resulted in a strain that plated with ∼100% efficiency under microaerobic conditions (Table 1). Our results therefore suggested a different function for this gene product in *B. fragilis*, prompting us to name the locus *oxe* (i.e., oxygen-enabled; Fig. 1).

To determine if mutations in other genes could give rise to O_2-enabled strains, a pool of 8,000 Tn*4400′* (30) transposon-insertion mutants was plated on brain heart infusion broth supplemented with 0.5% yeast extract and 15 μg/mL hematin (BHIS) under atmospheres containing as much as 2% oxygen. A PCR-based screen was used to determine if the enabled isolates had Tn*4400′* insertions in *oxe*. Of 32 analyzed isolates, 23 showed insertions of the IS element from Tn*4400′* within *oxe*. The other nine were found to fall into two groups that had insertions that lay outside of the *oxe* locus, but PCR amplification and sequencing of their *oxe* gene revealed that each had acquired spontaneous mutations in *oxe* in addition to the transposon

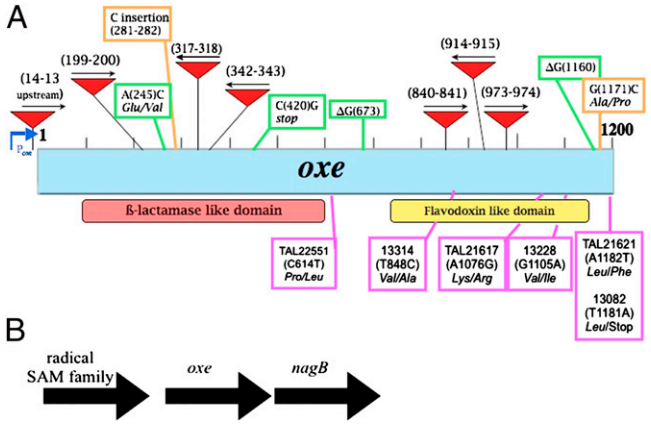

Fig. 1. Map of the *oxe* region of *B. fragilis*. (*A*) Diagram of the *oxe* gene (blue box). Predicted protein domains are shown below. Red arrows depict IS*4400′* insertions. Shown in the green boxes are the mutations found in spontaneous oxygen-enabled mutants of ADB77, with nucleotide positions in parentheses. Orange boxes denote mutations found in oxygen-enabled mutants from transposon-insertion pool. Purple boxes denote polymorphisms in Oxe sequence from clinical strains. (*B*) Genomic region surrounding the *oxe* gene. *nagB*, glucosamine 6-P deaminase.

insertion (Fig. 1). Importantly, introduction of a plasmid encoding WT *oxe* into these strains prevented growth under microaerobic conditions, indicating that the O_2-enabled phenotype was a result of the mutations in *oxe*. Additionally, four isolates that arose from microaerobic plating of the WT *B. fragilis* strain were sequenced across the *oxe* locus. All were found to contain mutations in *oxe* (Fig. 1).

Δ*oxe* Strain Plates at Unity in as Much as 2% Oxygen. Although both WT and Δ*oxe* strains plated with ∼100% efficiency in environments containing ≤0.05% oxygen compared with their respective anaerobic controls, they differed significantly in plating efficiencies at higher O_2 concentrations (Fig. 2). WT *B. fragilis* gave rise to colonies at a frequency of $\sim10^{-6}$ under 0.2% to 2% oxygen and failed to form detectable numbers of colonies in more oxygenated environments. The Δ*oxe* strain, however, gave rise to similar numbers of colonies in all O_2 levels as high as 2% (Fig. 2), and plated with a frequency of $\sim10^{-6}$ under 5% oxygen. Importantly, when a plasmid-borne copy of *oxe* was introduced into the Δ*oxe* strain, the increased plating efficiency with 2% O_2 was lost, indicating that the phenotype could be complemented (Fig. 2).

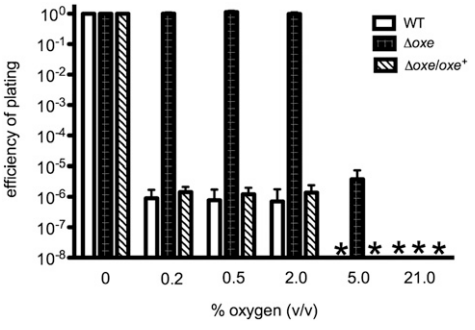

Fig. 2. Efficiency of plating under various percentages of oxygen. ADB77 (clear bars), ADB267 (black bars), and ADB267/pADB293 (hatched bars) were grown anaerobically to log phase and plated on BHIS under various oxygen concentrations. Procedure and calculations are as in Table 1. An asterisk indicates that values were below the limit of detection.

Δ*oxe* Strain Is Auxotrophic for Amino Acids Under Microaerobic Conditions. As shown in Fig. 3*A* and 4*A*, WT *B. fragilis* and the Δ*oxe* strain grow equally well under anaerobic conditions in rich and minimal medium. However, when BHIS cultures were shaken under 1% oxygen, the Δ*oxe* mutant began growing immediately whereas the WT exhibited a long lag phase lasting 20 to 30 h. Growth was similarly delayed for a Δ*oxe* strain carrying a WT copy of *oxe* on a plasmid, but not for one carrying an empty vector (Fig. 3*B*). When the WT and complemented strains had initiated growth, they doubled at approximately the same rate as the Δ*oxe* strain, and linear regression of the exponential growth rate suggested that this growth was a result of a variant population arising from a few bacteria present at the beginning of the experiment. Samples taken after 92 h of growth were clonally purified under anaerobic conditions, and isolates from this population were found to grow under microaerobic conditions, but failed to grow in room air. PCR amplification and sequence analysis of the *oxe* locus from six of these isolated colonies (three WT and three of the complemented strain) indicated that all had mutations within the *oxe* coding region. Thus, all the growth observed in BHIS with 1% O_2 was due to the presence of *oxe* mutants. In minimal medium, neither WT nor the Δ*oxe* strain grew when shaken under 1% oxygen, although supplementation with casamino acids restored some growth to the Δ*oxe* mutant but not to WT (Fig. 4*B*).

Δ*oxe* Retains Greater Viability Under Room Air than WT. Although the Δ*oxe* mutant clearly demonstrated a growth advantage vs. WT in a microaerobic environment, we wondered if the *oxe* deletion would increase aerotolerance when cells were shaken in room air. Fig. 5 shows that the Δ*oxe* strain retains greater viability than WT over several days of exposure in PBS solution with glucose, culminating in a ∼10-fold advantage after 48 h. The increased aerotolerance of the Δ*oxe* strain was lost when *oxe* was provided on a plasmid, but introduction of an empty vector had no effect.

Oxe Is Not a Major Source of ROS. *oxe* is predicted to encode a flavoprotein, a class of enzymes that uses flavins as cofactors 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 (1, 2). One model to explain the inability of WT *B. fragilis* to grow microaerobically, therefore, would posit that Oxe produces crippling amounts of ROS when cells are exposed to oxygen. To test this hypothesis, we deleted *oxe* from a strain with deletions in *katB*, *ahpC*, and *tpx*. We have previously shown (34) 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. When the Δ*kat*Δ*ahpC*Δ*tpx* strain was shaken under room air at an OD_{600} of 0.1, it accumulated H_2O_2 at a rate of 24 ± 1 nM/min. However, deleting *oxe* from this genetic background resulted in a strain that accumulated 29 ± 3 nM H_2O_2/min, a rate very similar to the parent strain, indicating that Oxe is not a major source of ROS under these conditions.

Δ*oxe* Strain Scavenges H_2O_2 More Efficiently than WT. In addition to AhpC and Tpx, there are four other peroxidases and two rubrerythrins 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 WT and Δ*oxe* strains by first shaking them aerobically and then adding exogenous peroxide to whole-cell suspensions. Fig. 6 shows that the Δ*oxe* mutant did indeed scavenge H_2O_2 more effectively than WT, and that this scavenging rate is reduced when a WT copy of *oxe* is provided *in trans*.

Spontaneous Oxygen-Enabled Mutants Arise in *Bacteroides 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 microaerobic conditions. We were able to isolate colonies of *B. thetaiotaomicron* American Type Culture Collection 29741 capable of growth on BHIS under 0.5% O_2 at a frequency of $\sim 10^{-7}$ relative to anaerobic controls. *B. thetaiotaomicron* encodes a protein with 97.2% homology to Oxe, and the gene is surrounded by loci encoding a putative radical SAM-family protein and NagB as in *B. fragilis*. 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 predicted Oxe gene products showed amino acid changes (R193H and S244I) relative to WT. The third isolate encoded a WT Oxe, but showed a predicted amino acid change (T322I) in the product of the *orf* upstream of *oxe*, indicating that this putative radical SAM-family protein may play a role in an Oxe-dependent pathway.

Clinical Isolates of *B. fragilis* Display Greater Range of Microaerobic Plating Efficiencies than Fecal Isolates. In the anaerobic environment of the mammalian intestine, *B. fragilis* is well protected from the toxic effects of oxygen. However, disruption of the intestinal wall can allow release of bacteria into more oxygenated tissue, posing a significant challenge to the survival of anaerobes. The peritoneum, for instance, contains 2% to 7% oxygen (35, 36). *B. fragilis* is capable of withstanding this microaerobic environment long enough to allow for the establishment of abscesses, which are eventually rendered anaerobic with the help of fac-

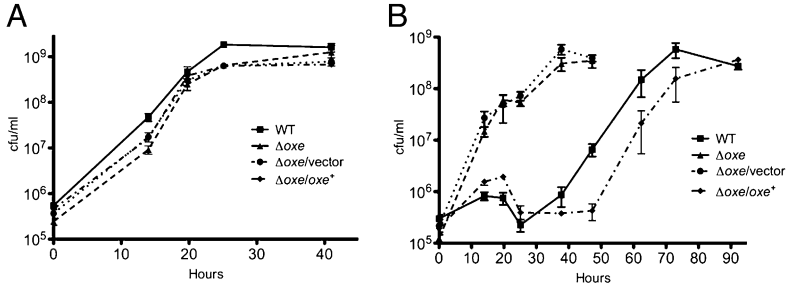

Fig. 3. Growth of *B. fragilis* Δ*oxe* strains in rich medium. ADB77 (■), ADB267 (▲), ADB267/pJST61 (●), and ADB267/pADB293 (◆) were grown anaerobically to late log-phase in BHIS with or without erythromycin. These cultures were diluted 1:1,000 into fresh medium and shaken in flasks at 200 rpm in an anaerobic chamber (*A*) or in a chamber containing 1% oxygen (*B*). Samples were diluted in PBS solution. Aliquots (10 μL) were spotted to prereduced BHIS plates (with or without erythromycin) and incubated in the anaerobic chamber. Counts (in cfu) were enumerated after 24 to 36 h incubation by using a stage microscope. Results shown are the means of three experiments ± SEM.

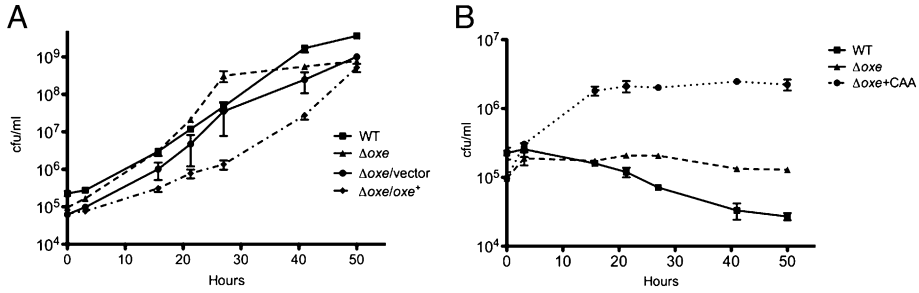

Fig. 4. The *B. fragilis* Δ*oxe* strain is auxotrophic for amino acids when grown microaerobically. ADB77, ADB267, ADB267/pJST61, and ADB267/pADB293 were grown anaerobically to late log phase in AMM with or without erythromycin. These cultures were diluted 1:2,000 into fresh medium and shaken in flasks at 200 rpm in an anaerobic chamber (*A*) or in a chamber containing 1% oxygen (*B*). Where appropriate, Casamino acids (CAA) were added to 200 μg/mL. Samples were diluted in PBS solution and plated on prereduced BHIS. Counts (in cfu) were enumerated after 24 to 36 h. Results shown are the means of three experiments ± SEM: (*A*) ADB77 (■), ADB267 (▲), ADB267/pJST61 (●), and ADB267pADB293 (◆); and (*B*) ADB77+CAA (■), ADB267 (▲), and ADB267+CAA (●).

ultative anaerobes like *Escherichia coli*. However, we wondered if the transition between intestine and abscess might supply selective pressure for "oxygen-enabled" variants of *B. fragilis*, thus leading to an enrichment of these variants in the abscess. To test this idea, we determined the microaerobic plating efficiency of 26 independent clinical isolates of *B. fragilis* and compared them vs. eight fecal isolates from healthy volunteers. Strains were obtained from four different collections, and clinical specimens were isolated from various sites of infection. Ten clinical isolates plated with efficiencies of $10^{-5.5}$ or less under 0.5% to 1% oxygen, similar to ADB77. However, five isolates gave rise to colonies at frequencies of 10^{-5} to 10^{-2}, and the remaining 11 plated with frequencies $>10^{-2}$. In contrast, none of the fecal isolates plated with efficiencies $>10^{-5.5}$.

The *oxe* gene from all strains was PCR amplified and sequenced. The predicted Oxe sequence of all fecal strains matched that of our reference strain, ADB77. Among those clinical strains that plated with low efficiency under microaerobic conditions, two encoded Oxe polymorphisms (V283A and V369I). Polymorphisms were also found in one clinical strain that plated with intermediate efficiency (L394stop), and three that grew with high efficiency (K359R, L394F, and P205L; Fig. 1*A*). Predicted Oxe sequences from the remaining strains matched ADB77.

Discussion

The discovery that *B. fragilis* can grow nanaerobically (21) suggested that its response to oxygen is more complex than previously thought. This complexity is further underscored by our finding that *B. fragilis* mutants capable of microaerobic growth can be readily isolated, and that all such oxygen-enabled mutants derived from *B. fragilis* TM4000 studied to date carry lesions in *oxe*. Clearly Oxe is not essential for anaerobic growth in vitro, yet microaerobic growth was partially dependent on the addition of Casamino acids. Earlier work with an *E. coli sod* mutant demonstrated a similar conditional sensitivity to oxygen (37), a phenotype later found to be the result of superoxide-mediated destruction of the iron-sulfur cluster of dihydroxyacid dehydratase, a key enzyme in the production of branched-chain amino acids (38). Therefore, the auxotrophies displayed by the Δ*oxe* mutant under microaerobic conditions suggest that, although this strain is capable of growth in the presence of oxygen, it may be experiencing significant oxidative stress.

The increased tolerance of the Δ*oxe* strain to room air was similar to results seen with a *D. vulgaris roo* mutant (33) despite the increased sensitivity of this *roo* strain to a microaerobic environment. Clearly, the response to oxygen is varied among anaerobes, a point emphasized in work with *Clostridium acetobutylicum* that showed that activation of its OSR via the deletion of the gene encoding the PerR repressor gave rise to a more aerotolerant strain capable of some growth in room air (39). This result would seem to contrast with our finding that up-regulation of the *B. fragilis* OSR via a constitutively active OxyR did not allow for growth under higher O_2 concentrations. Additionally, the authors note that this *perR* mutant exhibited a growth defect under anaerobic conditions, perhaps explaining why *perR* mutants do not arise spontaneously, unlike *B. fragilis oxe* mutants.

We have shown that Oxe is not a major source of ROS 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, unlike fumarate reductase (Frd), another flavin-containing enzyme that we have demonstrated accounts for ~47% of the ROS generated by aerated *B. fragilis* (34). Interestingly, a *frd* mutant does not grow microaerobically unless *oxe* is also inactivated, indicating that a substantial reduction in ROS production is not enough to give rise to an oxygen-enabled strain.

Although deleting *oxe* did not lower the rate of production of endogenously generated ROS, it did increase H_2O_2 detoxification, thus allowing the Δ*oxe* strain to more efficiently protect vulnerable metabolic enzymes under increased oxygen concentrations and thereby likely explaining the oxygen-enabled phenotype. The enhanced H_2O_2 detoxification is most likely not caused by the increased expression of peroxide scavenging enzymes, as quantitative PCR did not reveal any major differences in the expression of peroxide scavenging enzymes between WT and the Δ*oxe* strain. We therefore believe that the Δ*oxe* strain most likely accumulates excess reductant, and that

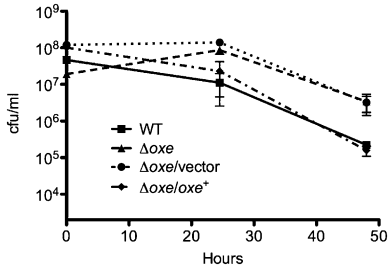

Fig. 5. *B. fragilis* Δ*oxe* is more aerotolerant than WT. ADB77 (■), ADB267 (▲), ADB267/pJST61 (●), and ADB267/pADB293 (◆) cultures were grown anaerobically in BHIS to midlog phase. Cells were pelleted and washed with PBS solution, and then resuspended in 10 mL PBS solution plus thymine and 0.5% glucose in 125-mL foam-stoppered flasks. Flasks were shaken at 37 °C under room air. Samples were serially diluted in PBS solution. Aliquots (10 μL) were spotted to prereduced BHIS plates (with or without erythromycin) and incubated in the anaerobic chamber. Counts (in cfu) were enumerated after 2 d incubation by using a stage microscope. Shown are the means of three experiments ± SEM.

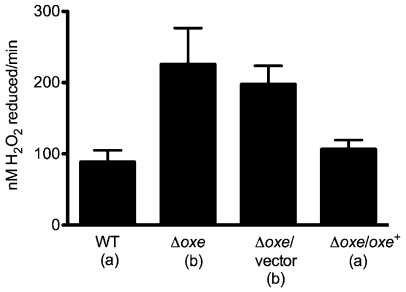

Fig. 6. *B. fragilis* Δ*oxe* strain scavenges H_2O_2 at a faster rate than WT. Strains were grown to log phase in AMM (with or without erythromycin), and cells were pelleted by centrifugation. Pellets were resuspended in AMM lacking cysteine and shaken at 37 °C under room air for 1 h. Cells were again pelleted, washed with PBS solution, and resuspended to an OD_{600} of 0.3 in PBS solution containing thymine and 0.5% glucose. H_2O_2 (5 μM) was added to start the assay. Samples were removed over time and centrifuged briefly, and supernatants were assayed for $[H_2O_2]$ by using Amplex Red. Rates are reported in nM H_2O_2 scavenged per minute. Shown are the means of at least three experiments ± SEM. ANOVA revealed a P value of 0.0066. Values for strains marked "a" differed significantly from those marked "b" with a $P < 0.05$ in a Newman–Keuls multiple comparison posttest.

peroxidases can tap into this reductant pool to more efficiently disproportionate H_2O_2. A similar model was proposed to explain the tolerance of a *roo* strain to room air (33), wherein the authors hypothesized that reduced rubredoxin normally consumed by the Roo-mediated reduction of oxygen might be funneled into ROS-scavenging reactions. Deletion of rubredoxin did not suppress the formation of oxygen-enabled mutants in our study (Table 1), so the identity of the key reductant is still unknown. However, the requirement of AhpC and Tpx for microaerobic growth suggests that NAD(P)H may play a critical role.

Our finding that clinical isolates of *B. fragilis* display a greater range of microaerobic plating efficiencies than fecal isolates suggests that passage from the intestine to oxygenated tissues might provide selective pressure for variants more capable of withstanding oxidative stress. Mutations in *oxe* might provide such an advantage, although the in vivo situation is obviously complex, as some enabled clinical strains did not have changes in Oxe sequence whereas others encoded polymorphisms in Oxe but did not plate with high efficiency under microaerobic conditions. The predicted changes in Oxe sequence in clinical strains are most likely not the result of laboratory manipulations made after isolation, as we have two clinical strains (TAL22551 and TAL22557) isolated from different sites in the same patient and both encode the same polymorphism (P205L), indicating that any potential mutations occurred in vivo. Additionally, clinical strains were passaged in the same manner as laboratory strains during the course of these experiments, yet ADB77 still plates at a frequency of $\sim 10^{-6}$ microaerobically.

The homology of Oxe to NorV (40) suggested that it might play a role in protection against nitric oxide. However, growth of WT and Δ*oxe* was inhibited by similar concentrations of the nitric oxide donor DEANONOate, and extensive amperometric studies suggested that these strains could reduce nitric oxide at similar rates. We therefore do not know the function of Oxe in the anaerobic physiology of *B. fragilis*, although it appears to play an important role in vivo, as *B. thetaiotaomicron* strains carrying transposons in the *oxe* gene are outcompeted in the gnotobiotic mouse intestine (41). It is therefore possible that *Bacteroides* acquired Oxe during its evolution as a mammalian commensal, and that a progenitor was capable of growth in much higher concentrations of oxygen. The fact that *B. fragilis* mutants capable of growth under 5% oxygen can also be isolated further suggests that evolution in an aerobic world may not have always proceeded linearly with respect to degrees of aerotolerance.

Materials and Methods

Reagents. H_2O_2 and horseradish peroxidase were purchased from Sigma, restriction enzymes were from New England Biolabs, and T4 DNA ligase and Amplex Red were from Invitrogen.

Growth Conditions. Anaerobiosis was maintained by using a Coy anaerobic chamber (Coy Laboratory Products) containing 85% nitrogen, 10% hydrogen, and 5% (vol/vol) CO_2. *B. fragilis* was grown in BHIS or anaerobic minimal medium (AMM) containing 0.5% glucose (42). In some cases, super-AMM plates containing 150 μg/mL hemin were used (43). For all *thyA* mutants, thymine was added to 50 μg/mL. Gentamicin (50 μg/mL), rifampicin (50 μg/mL), trimethoprim (80 μg/mL), erythromycin (8 μg/mL), and tetracycline (2.5 μg/mL) were added where appropriate. *E. coli* was grown aerobically in Luria broth, and chloramphenicol (25 μg/mL), ampicillin (100 μg/mL), and tetracycline (10 μg/mL) were added as appropriate.

Strains and Plasmids. Bacterial strains and plasmids used in this study are described in Table S1. *E. coli* strain DH5α was used for cloning, and strain HB101/RK231 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 (44). After isolation, fecal and clinical strains of *B. fragilis* were grown in anaerobic chambers or jars containing an anaerobic atmosphere. Passaging and maintenance of these strains was performed in the same manner as for the laboratory strains.

DNA Manipulation. Primers used in this study were synthesized by Integrated DNA Technologies and are listed in Table S2. Genomic sequence for *B. fragilis* NCTC9343 (GenBank accession no. CR626927.1) was provided by the Sanger Centre (www.sanger.ac.uk/Projects/B_fragilis) or by Pedant3 (http://pedant.gsf.de). Genomic DNA was amplified by using HotStarTaq Master Mix (Invitrogen). Plasmid and PCR product purifications were performed with QIAprep spin columns (Qiagen). Primers oxe1 and oxe4 were used in combination with primer 646J to amplify *oxe*::IS4400 junction fragments by using the PCR method. DNA sequencing was performed by the Tufts University Core Facility.

Strain Construction. All deletion mutants were constructed using a double-crossover technique as previously described (45). DNA fragments for cloning were obtained by PCR amplification of chromosomal DNA from strain ADB77. To build a deletion construct for *oxe*, primers oxe1 and oxe2 were used to amplify a fragment consisting of 724 bp of *oxe* upstream sequence and 86 bp of *oxe* amino-terminal coding sequence. The downstream fragment was created by PCR amplifying a region consisting of 44 bp of *oxe* carboxyl-terminal coding sequence and 836 bp of downstream sequence with primers oxe3 and oxe4. Purified PCR products were digested with HindIII/NcoI and NcoI/BamHI, purified, and ligated to pADB242 that had been digested with BamHI and HindIII to create pADB267. A similar scheme was used to build deletion constructs for *oxyR*, rubredoxin, and rubrerythrins with the appropriate primers.

The suicide plasmid pADB267 was delivered to the recipient *B. fragilis* strains as previously described (46). Tet^R colonies were screened for the appropriate cointegration event by using primers 61RAB and oxe5. Following recombination and resolution of the integrated plasmid, Tet^S colonies were screened via PCR with primers oxe5 and oxe4 to identify Δ*oxe* clones.

The *oxe* complementing plasmid was created by PCR amplifying a region from 270 bp upstream of the *oxe* translational start site to 77 bp downstream of the *oxe* coding sequence by using primers oxe6 and oxe7. The purified PCR product was digested with BamHI and ligated to BglII-digested pJST61 to create pADB293.

Growth Curves. Dense cultures of WT and Δ*oxe* strains were subcultured as indicated into fresh medium. When appropriate, casamino acids were added to 200 μg/mL. Cultures were grown at 37 °C with shaking (200 rpm) under anaerobic or microaerobic conditions (0.25–1% oxygen). Samples were serially diluted in reduced PBS solution or growth medium, and 10-μL aliquots were spotted on BHIS plates that had been reduced overnight in the anaerobic chamber. Counts (in cfu) were enumerated under a stage microscope after 1 to 2 d. Oxygen concentration was measured by using the pyrogallol method (21) or with a Coy oxygen analyzer ESD (model 630) and adjusted as necessary by addition of anaerobic gas mix.

Efficiency of Plating. *B. fragilis* strains were grown anaerobically to midlog phase in BHIS, serially diluted, and spread on BHIS plates that had been reduced in an anaerobic chamber overnight. Plates were transferred anaerobically to AnaeroPack boxes (Mitsubishi Gas Chemical) 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. O_2 concentrations described for experiments involving AnaeroPack boxes refer to that which was initially present. An *oxe*$^+$ strain was included in all experiments as a negative control to ensure that sufficient oxygen was retained in these boxes throughout the experiments. 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. O_2 concentrations were monitored throughout the course of the experiments and adjusted as necessary. Counts (in cfu) were enumerated after 3 to 5 d at 37 °C.

Room Air Challenge. Strains were grown in BHIS broth under anaerobic conditions until midlog phase. Cultures were centrifuged, and pellets were washed once with PBS solution. After a second centrifugation, pellets were resuspended in 10 mL oxygenated PBS solution, and 0.5% glucose and 50 μg/mL thymine were added. Samples were taken immediately and serially diluted, and a 10-μL aliquot was spotted onto prereduced BHIS plates (with or without erythromycin), which were incubated in the anaerobic chamber. Cell suspensions were transferred to 125-mL foam-stoppered flasks and shaken at 250 rpm and 37 °C under room air. Samples were processed as above. Counts (in cfu) were enumerated after 2 d of incubation.

H_2O_2 Detection. Cultures were grown anaerobically to midlog phase in AMM and treated as previously described (34). After shaking in room air, cells were washed twice and resuspended to an OD_{600} of 0.3 in 10 mL PBS solution containing 50 μg/mL thymine and 0.5% glucose. H_2O_2 (5 μM) was added to cell suspensions to start the assay, and the H_2O_2 concentration was followed over time by using Amplex red (34).

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