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Measurement of Predation and Biofilm Formation under Different Ambient Oxygen Conditions Using a Simple Gasbag-Based System

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***Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are Gram-negative bacteria characterized by predatory behavior. The aim of this study was to evaluate the ability of the predators to prey in different oxygen environments. When placed on an orbital shaker, a positive association between the rate of aeration and predation was observed. To further examine the effects of elevated ambient oxygen levels on predation, a simple gasbag system was developed. Using the system, we were able to conduct experiments at ambient oxygen levels of 3% to 86%. When placed in gasbags and inflated with air, 50% O_2, and 100% O_2, positive predation was seen on both planktonic and biofilm-grown prey cells. However, in low-oxygen environments, predatory bacteria were able to attack only prey cells grown as biofilms. To further evaluate the gasbag system, biofilm development of Gram-positive and Gram-negative microorganisms was also measured. Although the gasbag system was found to be suitable for culturing bacteria that require a low-oxygen environment, it was not capable of supporting, with its current configuration, the growth of obligate anaerobes in liquid or agar medium.**

Acute and chronic wounds are a major burden to public health and the economy (1). It is estimated that, in the United States alone, chronic wounds affect 6.5 million patients (2). One medical procedure used to promote wound healing is the use of oxygen therapy (3). For example, the use of oxygen under pressure or "hyperbaric oxygen therapy" (HBO_2) was shown to have a positive effect on ischemia and local wound hypoxia (4–6). In the last few years, additional medical procedures that allow topical administration of oxygen therapy directly to the wound site in order to support wound healing have been developed (7–9). One of the major complications affecting wound healing are infection, many of which are caused by multidrug-resistant pathogens and surface-associated (biofilm) bacteria (1, 10–12). It was previously suggested that predatory bacteria from the genus *Bdellovibrio* and *Micavibrio* could be used as live antibiotics to control wound infections (13, 14).

The purpose of this study was to evaluate the ability of predatory bacteria to prey in elevated ambient O_2 levels with the long-term goal of using predatory bacteria in combination with traditional oxygen therapy in order to enhance wound healing. To this end, a new gasbag system that allowed experiments to be conducted in diverse gas environments was developed. The system was also used to evaluate biofilm formation of medically relevant Gram-positive and Gram-negative bacteria in O_2 environments that were not examined previously.

MATERIALS AND METHODS

Predatory bacteria. The predatory bacteria used in this study were *Micavibrio aeruginosavorus* ARL-13 (15, 16), *Bdellovibrio bacteriovorus* 109J (ATCC 43826), *B. bacteriovorus* HD100 (17, 18), and a previously isolated host-independent (HI) variant of *B. bacteriovorus* 109J (19). The HI variant was cultured at 30°C in peptone-yeast extract (PYE) (10 g/liter peptone and 3 g/liter yeast extract amended with 3 mM $MgCl_2$ and 2 mM $CaCl_2$). Predatory bacteria were enumerated as PFU (13, 20).

Predator stock lysates were prepared by coculturing the predators with prey bacteria in diluted nutrient broth (DNB) (13). The cocultures were incubated on a rotary shaker at 30°C. *B. bacteriovorus* was cocultured on *Escherichia coli* strain S17-1 (21) or ZK2686 (22). *M. aeruginosavorus* was cultured on *Pseudomonas aeruginosa* UCBPP-PA14 (PA14) (23). Fresh predator cultures were prepared in 20 ml DNB supplemented with 2 ml of overnight prey cells (~1×10^9 CFU/ml) and 2 ml of predatory bacteria, which was removed from a premade stock lysate. The cocultures were incubated at 30°C for 24 h. Thereafter, the lysates were passed through a 0.45-μm-pore-size Millex-HV filter (Millipore, Billerica, MA) to remove any residual prey cells (harvested predator) (13).

Predation experiments. (i) Predation on planktonic cells. Cocultures were prepared by adding 1 ml of harvested predators (~1×10^8 PFU/ml) to 1 ml of DNB-washed prey cells (~1×10^9 CFU/ml) and 8 ml DNB medium. Cultures were incubated at 30°C. Predation was measured by the reduction in prey cell viability measured by dilution plating and CFU enumeration (13). Experiments were conducted three times and yielded similar results.

(ii) Predation on biofilms. Predation on surface-attached cells was done as described previously (24, 25). Briefly, biofilms were developed as shown below and washed with DNB to remove nonattached cells. One hundred microliters of harvested predator was added to each well. Predator-free medium was used as a control. The plates were incubated at 30°C. *E. coli* strain ZK2686 and *P. aeruginosa* PA14 were used as prey for *B. bacteriovorus* and *M. aeruginosavorus*, respectively.

To measure biofilm reduction, the wells were stained with 0.1% crystal violet (CV). CV was solubilized using 50% acetic acid. Relative biofilm biomass was assayed by measuring the absorbance of the CV solution at 600 nm (A_{600}) (26).

Biofilm assay. Overnight cultures were diluted 1:100 in fresh medium and inserted in 96-well microtiter wells (100 μl per well). Plates were incubated for 24 h before being stained with CV (26, 27). The Gram-negative bacteria used were *E. coli* strain ZK2686, *P. aeruginosa* PA14, and *Serratia marcescens* 361 (Presque Isle Cultures, Erie, PA). An additional eight keratitis clinical isolates of *P. aeruginosa* and nine keratitis clinical isolates of *S. marcescens* were also examined. Clinical isolates were kindly provided by R. Shanks (Campbell Laboratory of Ophthalmic Microbiology, Department of Ophthalmology, University of Pittsburgh Medical

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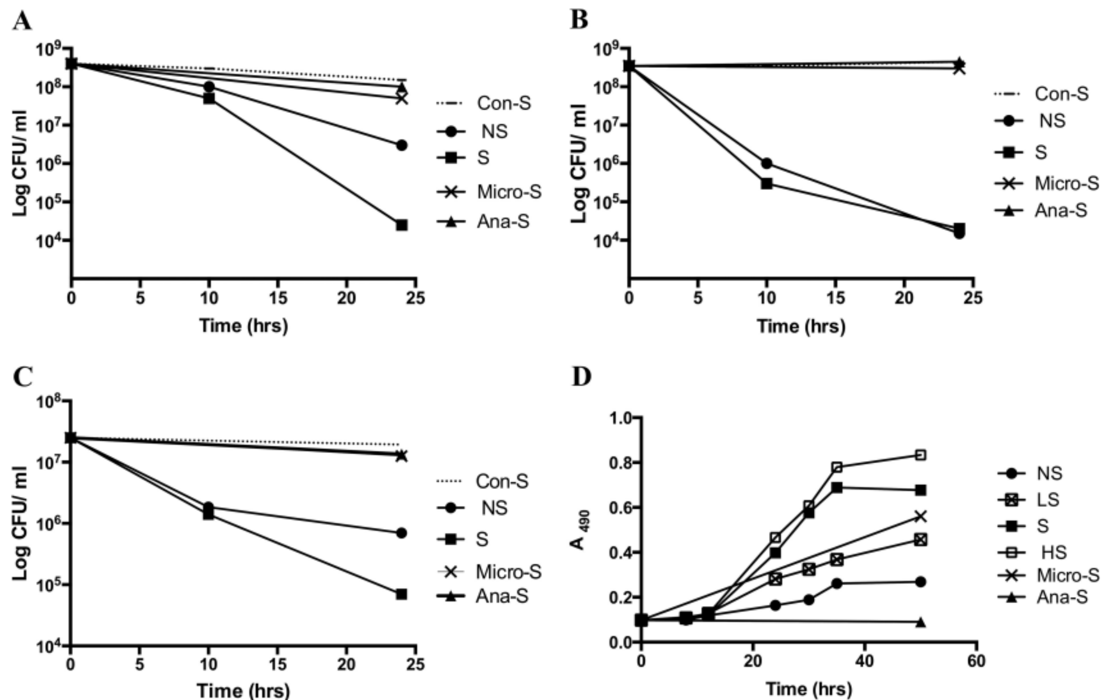

FIG 1 Effects of agitation on predation and *B. bacteriovorus* HI growth. (A to C) Cocultures of *B. bacteriovorus* 109J and *E. coli* (A), *B. bacteriovorus* HD100 and *E. coli* (B), and *M. aeruginosavorus* ARL-13 and *P. aeruginosa* (C) were placed at 30°C with no agitation (no shaking [NS]) or on an orbital shaker set at 120 rpm/min (shaking [S]). Cultures were also placed in anaerobic GasPak jars (Ana-S) and microaerophilic GasPak jars (Micro-S). Predator-free culture was used as a control with shaking (Con-S). Predation was measured by the reduction in prey cell viability. (D) The *B. bacteriovorus* HI variant was placed in PYE medium at 30°C with no agitation (NS) or on a shaker set at 32 rpm/min (LS), 120 rpm/min (S), and 250 rpm/min (HS). The cultures were also placed in anaerobic GasPak jars (Ana-S), microaerophilic GasPak jars (Micro-S), and unsealed jars (S). Growth was measured by the change in culture turbidity measured at 490 nm (A_{490}).

Center [UPMC]). *E. coli* and *S. marcescens* biofilms were grown in LB medium. *P. aeruginosa* biofilms were grown in 20% King's-B medium (25). Biofilms were grown at 30°C. The Gram-positive bacteria used were *Staphylococcus aureus* SH1000 (28) and *Staphylococcus epidermidis* NJ9709 (29). Biofilms were grown in tryptic soy broth (TSB) supplemented with 0.6% yeast extract and 0.8% glucose (28, 29). Gram-positive and Gram-negative biofilms were grown at 37°C and 30°C, respectively.

Reduced oxygen environments. For anoxic growth environment, plates and cultures were placed in a magnetic activated cell sorting (MACS) MG 250 anaerobic chamber (Microbiology International, Frederick, MD) using anoxic gas mix (10% CO_2, 10% H_2, and 80% N_2). For culturing bacteria under anoxic or low-oxygen conditions with agitation, cultures were inserted into a BD GasPak jar system with a disposable anaerobic or microaerophilic gas-generating envelope (BD Diagnostic Systems, Franklin Lakes, NJ) and placed on an orbital shaker. The microaerophilic and anaerobic bacteria used were *Campylobacter jejuni* ATCC BAA-1153, *C. jejuni* ATCC 33560, *Fusobacterium nucleatum* ATCC 10953, *Fusobacterium necrophorum* PK1594, *Porphyromonas gingivalis* W83/ATCC BAA-308, and *Prevotella intermedia* ATCC 25611 (30–34). *C. jejuni* bacteria were cultured on *Brucella* medium. Anaerobic bacteria were cultured in TSB supplemented with 5 μg/ml hemin and 1 μg/ml menadione or TSB blood agar plates supplemented with 5 μg/ml hemin and 1 μg/ml menadione. Cultures were grown at 37°C.

Gasbag system. VWR sterile sample bags (VWR, Radnor, PA) were used. The bags are made from flexible, leak-proof, and highly resistant virgin polyethylene. Each bag is sterile and sealed with a tear-off perforated top and has a wire for closure. For liquid cultures, a sterile 650-ml bag was used (catalog number 89085-528; VWR). A 16-gauge 1.5-in. needle was inserted below the perforated top. Five milliliters of preinoculated growth medium was injected into the bags using a 5-ml syringe via the preinserted needle. Thereafter, the bags were inflated with the desired gas mixture using a clear polyvinyl chloride (PVC) tube (Fisher Scientific, Pittsburg, PA). The tube was connected to a gas cylinder on one side and to the preinserted needle on the other side. The needle was removed, and the bags were heat sealed below the needle insert point using a Creative 12-in. PFS-300 hand impulse plastic bag sealer. The heat gauge on the sealer was placed at position 3.5. The inflated bags, which look like inflated Ziploc bags, were placed on an orbital shaker and placed in a 30°C or 37°C incubator.

For experiments done with microtiter dishes or petri dishes, sterile 1,650-ml bags were used (catalog number 89085-554; VWR). In this configuration, the perforated top was removed, and the plates were inserted into the bags. The bags were heat sealed using the plastic bag sealer. A 16-gauge needle was inserted below the sealing. The needle was connected to a standard lab vacuum gas tap via a tube, which allowed the removal of trapped air. The tube was then connected to a gas cylinder, and the bags

TABLE 1 Ambient O_2 levels in the gasbag system[a]

Gas composition injected	Initial O_2 concn (%) measured in bag	Final O_2 concn (%) measured after 24 h
100% O_2	89	86
50% O_2 and 50% N_2	47	46
Ambient air	20	19
Anaerobic mix (80% N_2, 10% CO_2, and 10% H_2)	2.7	3.2
100% N_2	3	3.2

[a] The oxygen meter was inserted into a sterile sample bag. The bag was heat sealed, and the desired gas was inserted into each bag. The bag was heat sealed again and placed in an incubator at 30°C. The O_2 levels at time zero and at 24 h were measured using the preinserted O_2 meter.

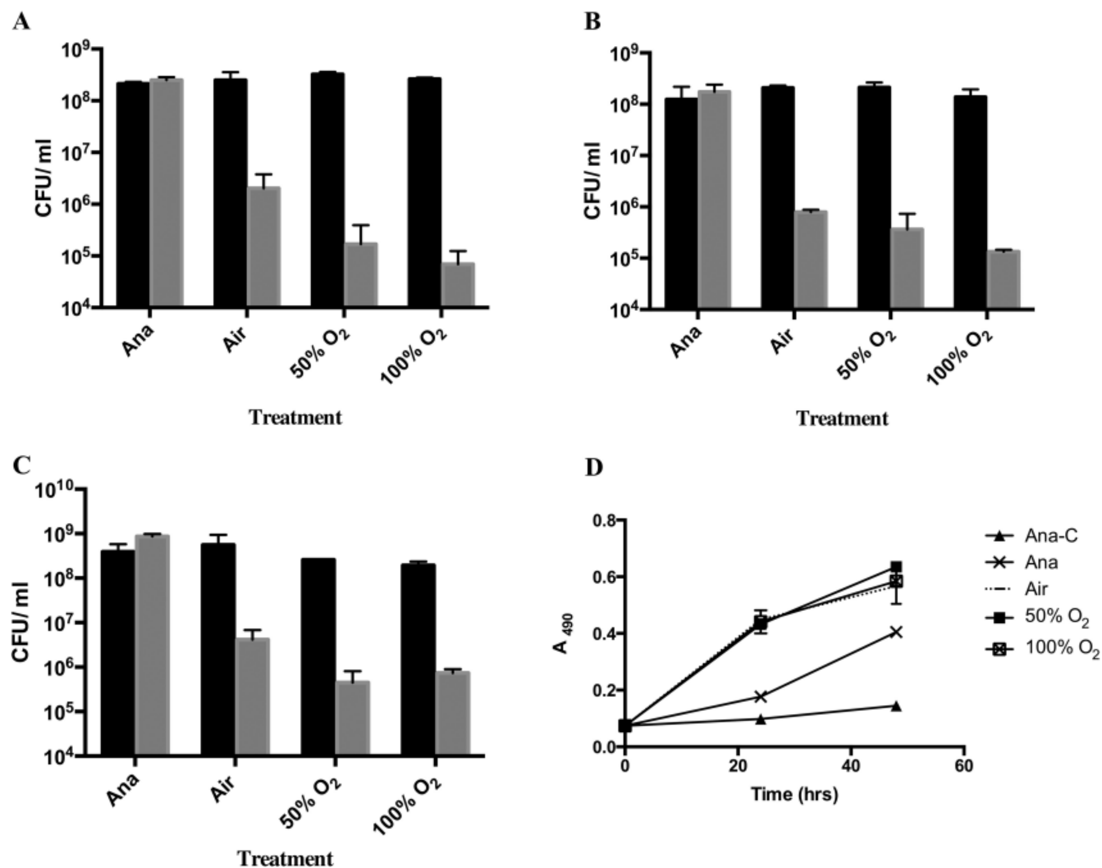

FIG 2 Predation and *B. bacteriovorus* HI growth in a gasbag system under different O_2 environments. (A to C) Cocultures (gray bars) of *B. bacteriovorus* 109J and *E. coli* (A), *B. bacteriovorus* HD100 and *E. coli* (B), and *M. aeruginosavorus* ARL-13 and *P. aeruginosa* (C) were injected into the sterile sample bags. The bags were inflated with the desired gas (Treatment), sealed, and placed on an orbital shaker set at 120 rpm/min at 30°C. Predator-free culture was used as a control (black bars). Predation was measured by the reduction in prey cell viability after 24 h of incubation. The gases used were anoxic mixture (Ana), ambient air (Air), 50% O_2, and 100% O_2. (D) The *B. bacteriovorus* HI variant was suspended in PYE medium and injected into sterile sample bags. The bags were inflated with the required gas, sealed, and placed on an orbital shaker set at 120 rpm/min at 30°C. Growth was monitored by the change in culture turbidity measured at 490 nm (A_{490}). The gases used were anoxic gas mixture (Ana), ambient air (Air), 50% O_2, and 100% O_2. The cultures were also placed in an anaerobic chamber (Ana-C). Each experiment was conducted in triplicate, with each value representing the mean and standard deviation (error bar).

were inflated. Finally, the bags were resealed below the needle insert point using the bag sealer. The bags were then placed in a 30°C or 37°C incubator.

The compressed gas cylinders used were filled with anoxic gas mixture (80% N_2/10% CO_2/10% H_2), 50% O_2 (50% O_2/50% N_2), 100% O_2, and 100% N_2. Gas cylinders were purchased from GTS-WELCO (Newark, NJ). Each cylinder was fitted with a regulator and a 0.45-μm filter. Ambient air was also used and inserted into the bags using a 60-ml syringe.

Yeast agglutination assay. Fimbria-dependent aggregation of *S. marcescens* to yeast cells was performed as described previously (35, 36). In brief, overnight cultures of *S. marcescens* were suspended in phosphate-buffered saline (PBS) buffer (optical density at 600 nm [OD_{600}] of 1.0). Five hundred microliters of the culture was mixed with 500 μl of yeast solution (Sigma YSC2; 2% [wt/vol] in PBS) in a 2-ml tube. The mixture was agitated for 1 min on an orbital shaker and allowed to stand at room temperature for 5 min. Three 100-μl samples were removed from the top aqueous layer of the tube, and the absorbance (OD_{600}) was measured. Thereafter, the tubes were vortexed for 30 s at high speed, three 100-μl samples were immediately removed from each tube, and the A_{600} was determined once more. The percentage of agglutination was determined as follows: $100 \times (1 - OD_{600}$ before vortexing/OD_{600} after vortexing) (37). Experiments were conducted in triplicate.

RESULTS

Agitation affects predation. In order to examine the effect of agitation on predation, cocultures were prepared in 125-ml flasks. Flasks were not agitated or were placed on an orbital shaker set at 120 rpm/min. Our assumption was that agitating the flasks would produce a more aerated environment. Cocultures were also placed in anaerobic and microaerophilic GasPak jars and placed on an orbital shaker. To ensure that the experimental outcome is not specific to a particular strain of *B. bacteriovorus*, we conducted the experiments with both the 109J and HD100 strains. As seen in Fig. 1A to C, the ability of the predators to reduce the prey was more effective under shaking conditions compared to the nonagitated cocultures. No decrease in prey cell viability was measured in agitated flasks placed in anoxic and low-oxygen conditions.

In order to further examine the effect of aeration on the growth of *Bdellovibrio*, an HI variant of *B. bacteriovorus* was cultured in PYE medium with different shaking conditions. In general, a positive association between the growth of the HI variant and the putative aeration conditions was seen (Fig. 1D). However, HI

growth was also measured in cultures that were placed in low-oxygen conditions (Fig. 1D, ×).

Developing a gasbag culturing system. To further investigate the effect of elevated O_2 concentrations on predation, a new system that allowed the use of high O_2 concentration in a laboratory setting was developed. To measure the O_2 concentration in the bag, a preliminary experiment was conducted in which a traceable, portable dissolved oxygen meter (Fisher Scientific, Pittsburgh, PA) was inserted into a sterile sample 1,650-ml bag through the wired side of the bag. The bags were then placed in an incubator set at 30°C for 24 h, and the ambient O_2 levels within the bags were measured using the preinserted O_2 meter. As seen in Table 1, the gasbag was able to maintain the initial O_2 concentration. In our experiments, we were unable to obtain 100% or 0% O_2.

Measuring predation in the gasbag system. To measure predation at different ambient O_2 concentrations, cocultures were prepared and injected into the 650-ml sterile sample bags. The bags were inflated with the selected gas, sealed, and placed on an orbital shaker. As seen in Fig. 2A to C, all predators were able to prey in bags inflated with air, 50%, and 100% O_2. Higher predation was measured in bags that were inflated with 50% and 100% O_2. No predation was measured in bags inflated with anoxic gas.

To investigate the ability of the predators to remove biofilms at different ambient O_2 concentrations, biofilms were developed and placed in the gasbags with or without the predators (control). As seen in Fig. 3, all of the predators were able to prey on the biofilms with improved predation occurring at higher ambient O_2 levels (Fig. 3A). Strikingly, although predatory bacteria were unable to prey on planktonic prey cells that were placed in low-oxygen conditions (Fig. 2A to C, Ana), biofilm-grown prey cells were not immune to predation under the same growth conditions (Fig. 3A, Ana-GB). To further examine this finding, biofilms were placed in an anaerobic chamber and in a GasPak jar under anoxic and low-oxygen conditions. As seen in Fig. 3B, positive predation did occur on biofilms placed in a low-oxygen, but not in an anoxic, environment.

Measuring biofilm development in a gasbag system. To measure biofilm formation by a variety of opportunistic pathogens at different ambient O_2 concentrations, bacteria were placed in 96-well plates and placed inside the bags. The bags were sealed and inflated with the desired gases. Plates were also placed in an anaerobic chamber. For *S. aureus* and *E. coli*, a positive correlation between the O_2 levels and biofilm formation was measured, with the most robust biofilm buildup seen in plates that were placed in bags inflated with 100% O_2. In contrast, *S. epidermidis* biofilm development spiked at ambient air oxygen levels (Fig. 4A). As was seen for *E. coli*, other Gram-negative bacteria also exhibited an increase in biofilm formation at elevated O_2 levels. Six out of the 9 *P. aeruginosa* strains tested showed higher biofilm buildup in gasbags inflated with 100% O_2 compared to the biofilm buildup seen in plates incubated with ambient air (Fig. 4B). For *S. marcescens*, 80% of the strains showed an increase in biofilm formation when placed in gasbags inflated with 50% O_2 (Fig. 4C).

***S. marcescens* fimbria activity assay.** Previous studies with *S. marcescens* have demonstrated a strong correlation between biofilm formation and fimbria activity (35, 36). One indirect method to measure fimbria activity is by agglutination to *Saccharomyces cerevisiae* (38, 39). To measure whether the enhanced biofilm formation seen at higher O_2 levels (Fig. 4C) might be associated with

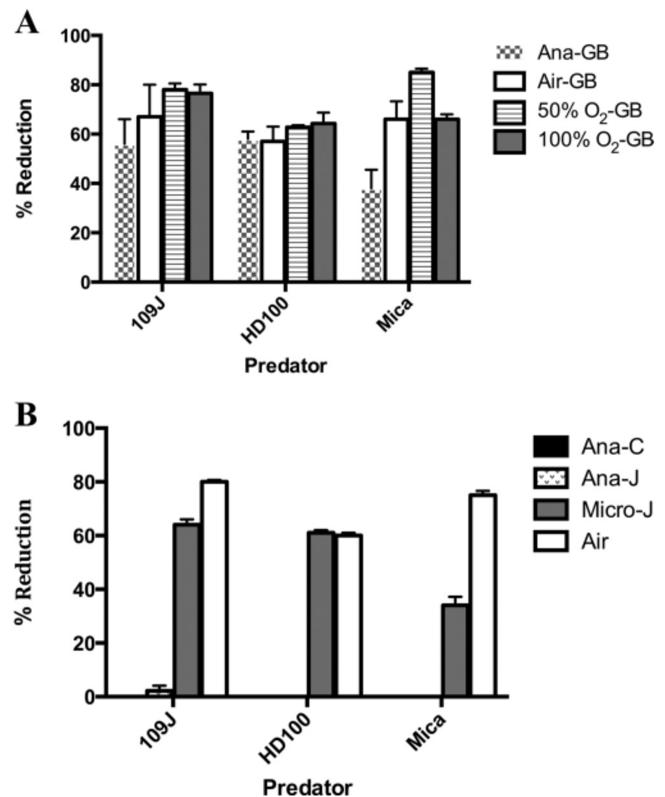

FIG 3 Predation of predatory bacteria on biofilms in different ambient oxygen levels. (A) Predation in a gasbag system. Preformed biofilms of *E. coli* and *P. aeruginosa* were treated with *B. bacteriovorus* 109J, *B. bacteriovorus* HD100, and *M. aeruginosavorus* ARL-13 (Mica). The bags were inflated with anoxic gas mixture (Ana-GB), ambient air (Air-GB), 50% O_2 (50% O_2-GB), and 100% O_2 (100% O_2-GB). After 24 h of incubation, the wells were washed and stained with CV, and the amount of staining was measured at 600 nm (A_{600}). Data represent the percent change in CV staining compared to biofilms that were treated with a predator-free control. (B) Predation in nongasbag systems. Preformed biofilms were inoculated with the predators and placed in an anaerobic chamber (Ana-C), anaerobic GasPak Jar (Ana-J), microaerophilic GasPak jar (Micro-J), and standard incubator (Air). Data represent the percent change in CV staining compared to biofilms that were treated with a predator-free control. Experiments were conducted with 16 wells for each treatment. Data show the mean plus standard deviation (error bar).

higher fimbria activity, a yeast agglutination test was conducted. *S. marcescens* strain K1885 was cultured overnight in gasbags inflated with air or 50% O_2. This isolate was selected for its elevated biofilm formation measured at high O_2 levels. No difference in growth was seen after 24 h of incubation (with cultures reaching OD_{600}s of 1.02 and 1.07 for air and 50% O_2, respectively). When mixed with the yeast, large macroaggregates were seen within 1 min of incubation in *S. marcescens* that was cultured in the high-oxygen environment, whereas only small microaggregates were seen in *S. marcescens* that were precultured in gasbags inflated with ambient air (data not shown). Five minutes postincubation with the yeast, 49.9% ± 1.2% of the *S. marcescens* cultured in high O_2 were aggregated, with only 7.7% ± 1.1% of the *S. marcescens* cells cultured with ambient air were in an aggregated state.

To examine whether the gasbag system could also be utilized for studies that require anoxic and low-oxygen environments, bacteria known to grow only under these conditions were examined. By inflating the bags with an anoxic gas mixture, we were

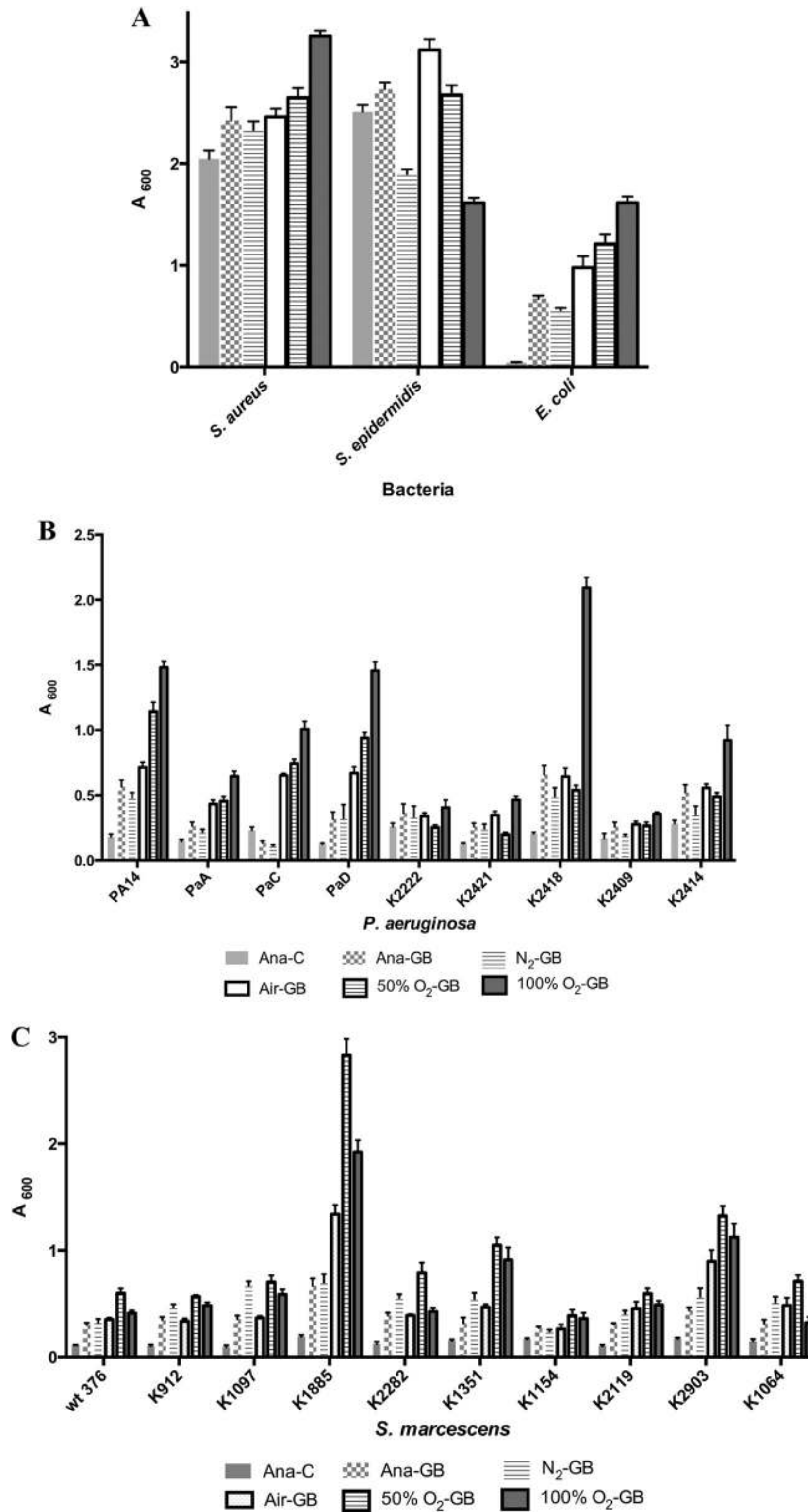

FIG 4 Effects of ambient oxygen levels on biofilm formation. (A to C) The wells of 96-well plates were inoculated with *S. aureus*, *S. epidermidis*, and *E. coli* (A), 9 strains of *P. aeruginosa* (B), and 10 strains of *S. marcescens* (C). The plates were placed in bags that were inflated with anoxic mixture (Ana-GB), 100% N_2 (N_2-GB), ambient air (Air-GB), 50% O_2 (50% O_2-GB), and 100% O_2 (100% O_2-GB). The plates were also placed in an anaerobic chamber (Ana-C). Data represent the amount of CV staining following 24 h of incubation and measured at 600 nm (A_{600}). Each value represents the mean of 12 wells. Error bars are shown as 1 standard deviation.

TABLE 2 Growth of microaerophilic and anaerobic bacteria in liquid cultures and agar plates

Bacterial species and strain	Growth[a] of bacteria in:					
	Anaerobic chamber		Bag inflated with anoxic gas mix		Bag inflated with air	
	Liquid (%)[b]	Plate[c]	Liquid (%)	Plate	Liquid (%)	Plate
C. jejuni strains						
ATCC BAA-1153	NA	NA	688	+	0	−
ATCC 33560	NA	NA	388	+	4	−
F. necrophorum strains						
PK1594	833	+	166	+	1	−
ATCC 10953	717	+	0	+/−	0	−
P. gingivalis W83/ATCC BAA-308	754	+	0	+/−	0	−
Prevotella intermedia ATCC 25611	NA	+	NA	+/−	NA	−

[a] Growth of microaerophilic and anaerobic bacteria in broth (liquid) and agar plates. Cultures were inserted in gasbags and inflated with anoxic gas mix or air. Anaerobic bacteria were also cultured in an anaerobic chamber as a positive control.
[b] Data represent the percent increase in turbidity compared to the initial inocula following 48 h of incubation. NA, not applicable.
[c] Data represent growth (+), slow growth (+/−), or no colony growth (−) on agar plates. The plates were incubated for up to 4 days. NA, not applicable.

able to facilitate the growth of the microaerophilic *C. jejuni*, both in broth and on agar plates (Table 2). However, we were unable to achieve the 0% O_2 conditions necessary to culture the obligate anaerobes *Porphyromonas gingivalis* and *Prevotella intermedia*, with only limited success in growing one strain of *F. necrophorum* (Table 2).

DISCUSSION

It was previously determined that *B. bacteriovorus* and *M. aeruginosavorus* are able to survive but not prey under anoxic and low-oxygen growth conditions (13, 30, 40). On the basis of this observation, we hypothesized that predation might be influenced by the oxygen levels in the coculture. In order to examine our hypothesis, predatory bacterial cocultures were placed at different agitation rates with the assumption that agitating the flasks would produce a more aerated environment. A positive association between the rate of agitation and predation was observed. The inability of the predators to reduce the prey numbers in anaerobic and microaerophilic jars, which were also agitated, strengthened our assumptions that the increase in predation is a result of improved aeration and not caused simply by stirring the culture. The inability of the predators to attack in anoxic and low-oxygen conditions is in agreement with a previous study in which the predators were cultured in similar conditions (13, 30). The ability of predatory bacteria to survive periods of anoxia and low-oxygen conditions was also demonstrated for intraperiplasmic and attack phase bdellovibrios (40, 41). Despite the fact that *B. bacteriovorus* was unable to prey in low-oxygen conditions, the HI variant was able to grow in flasks placed in the microaerophilic jars. We have previously shown that HI variants are capable of forming biofilms (42). Thus, it would be tempting to speculate that the ability of the HI variants to grow in low-oxygen conditions might allow it to grow in a biofilm where the O_2 levels are believed to be lower than the surrounding environment (43, 44).

In order to further study the effect of elevated O_2 levels on predation, a simple gasbag culturing system was developed. Using an O_2 meter, we have established that the gasbag is capable of maintaining reasonably stable O_2 levels. However, as some residual air remained trapped in the bag, we were unable to reach 100% O_2. Furthermore, with no catalyzing element to remove the residual oxygen, we were unsuccessful in obtaining full anoxic conditions. Our inability to achieve full anoxic conditions was confirmed by the fact that we were unable to culture *P. gingivalis* and *P. intermedia*. However, we were able to facilitate *C. jejuni* growth, a bacterium that requires a low-oxygen environment.

When placed on biofilms, all of the examined predators were able to prey on the biofilms, with improved predation occurring at higher ambient O_2 levels. Interestingly, positive predation was seen on biofilms that were placed in gasbags inflated with anoxic gas as well as on biofilms placed in GasPak jars under low-oxygen conditions. As the tentative ambient O_2 levels reached in the gasbags inflated with anoxic gas was around 3%, we conclude that predatory bacteria are able to attack biofilms, but not planktonic prey cells, in low-oxygen environments. Although high O_2 levels were found to stimulate predation on both biofilms and planktonic cells, additional experiments need to be conducted in order to determine whether oxygen therapy might be used to enhance the biological-control aptitude of predatory bacteria *in vivo*.

To establish additional uses for the gasbag system, we cultured bacteria in 96-well plates and examined their ability to form biofilms at different gas compositions. As expected, the ability of each bacterium to form a biofilm at different ambient O_2 levels was seen. Using the gasbag system, we were also able to conduct yeast aggregation experiments, which suggested that the elevated biofilm formation measured for *S. marcescens* might be linked to an increase in fimbria activity resulting from growth in a high-O_2 environment.

Although biofilm formation under low-oxygen conditions was measured for many of the bacteria examined in this study (45–49), the new gasbag system allows measuring biofilm growth and physiology under gas conditions that are not easily attained using, for the most part, standard lab equipment. The gasbag system might also facilitate studies that are aimed at understanding the effect of oxygen therapy on microbial physiology and pathogenesis (50–52).

In this study, we have developed a new, inexpensive, and versatile system that allows experiments to be conducted in a variety of ambient gas environments. As the system is relatively airtight, it could be operated with gases that are considered unsafe for use in a standard laboratory environment, such as high O_2 levels. With the profile of the inflated gasbag being small, it could be used with "standard" lab equipment such as incubators and shakers. Since the initial bag is sterile, medium, cultures, and samples could be inserted or injected directly into the bag without the risk of contamination or the need to presterilize the bag. Finally, the ability to insert liquids, petri dishes, and standard 96-well plates into the bag allows experiments to be conducted by standard methods. At this point, we are working on improving our system to a configuration

that will allow the removal of all trapped air and the use of materials that are more efficient in reducing gas diffusion.

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