

Video Article

***Porphyromonas gingivalis* as a Model Organism for Assessing Interaction of Anaerobic Bacteria with Host Cells**

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Abstract

Anaerobic bacteria far outnumber aerobes in many human niches such as the gut, mouth, and vagina. Furthermore, anaerobic infections are common and frequently of indigenous origin. The ability of some anaerobic pathogens to invade human cells gives them adaptive measures to escape innate immunity as well as to modulate host cell behavior. However, ensuring that the anaerobic bacteria are live during experimental investigation of the events may pose challenges. *Porphyromonas gingivalis*, a Gram-negative anaerobe, is capable of invading a variety of eukaryotic non-phagocytic cells. This article outlines how to successfully culture and assess the ability of *P. gingivalis* to invade human umbilical vein endothelial cells (HUVECs). Two protocols were developed: one to measure bacteria that can successfully invade and survive within the host, and the other to visualize bacteria interacting with host cells. These techniques necessitate the use of an anaerobic chamber to supply *P. gingivalis* with an anaerobic environment for optimal growth.

The first protocol is based on the antibiotic protection assay, which is largely used to study the invasion of host cells by bacteria. However, the antibiotic protection assay is limited; only intracellular bacteria that are culturable following antibiotic treatment and host cell lysis are measured. To assess all bacteria interacting with host cells, both live and dead, we developed a protocol that uses fluorescent microscopy to examine host-pathogen interaction. Bacteria are fluorescently labeled with 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and used to infect eukaryotic cells under anaerobic conditions. Following fixing with paraformaldehyde and permeabilization with 0.2% Triton X-100, host cells are labeled with TRITC phalloidin and DAPI to label the cell cytoskeleton and nucleus, respectively. Multiple images taken at different focal points (Z-stack) are obtained for temporal-spatial visualization of bacteria. Methods used in this study can be applied to any cultivable anaerobe and any eukaryotic cell type.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53408/>

Introduction

Anaerobic bacteria colonize almost all surfaces of the human body. Although predominant in the flora of the intestinal and genitourinary tracts where oxygen concentrations are low, they also exist at high levels on the skin, mouth, nose, and throat¹. Anaerobic bacteria are a common cause of endogenous infections and are frequently isolated from diseased sites. However, because of their fastidious nature, anaerobes can be difficult to isolate and culture. Studies involving anaerobic bacteria must be done under restricted conditions. Modern anaerobic-culture techniques allow researchers to mimic the anaerobic settings required to study many anaerobic laboratory strains or even clinical isolates^{2,3}.

Pathogenic anaerobic bacteria have developed a dynamic relationship and co-evolution with the host cells in which they reside. Most anaerobes are susceptible to killing by the host immune response before reaching infectious levels. However, some pathogenic bacteria have developed mechanisms to escape from or subvert the host immune response. They accomplish this goal through mechanisms such as evasion of immune recognition, neutralization of immune mediators, alteration of cell-mediated immunity, invasion of host cells, and alteration of immune signaling⁴. *Porphyromonas gingivalis*, a Gram-negative anaerobe implicated in both oral and extraoral diseases, is one example of a highly adapted bacterial pathogen capable of causing pathogenic changes in the host⁵⁻⁷.

Pockets of biofilm plaque accrued in deep crevices formed between the teeth and gingival mucosal tissue can harbor anaerobic bacteria that are protected from atmospheric oxygen⁸. These periodontal pockets serve as a niche for various anaerobic pathogens, such as *P. gingivalis*⁹. *P. gingivalis* is a keystone pathogen that is capable of remodeling the oral microbial community in ways that promote development and progression of periodontal diseases¹⁰. It produces a large number of virulence factors that are active against a broad spectrum of host proteins and provides mechanisms for evasion of host defenses¹¹. It is also capable of invading epithelial, endothelial, fibroblastic, and periodontal ligament cells *in vitro*¹²⁻¹⁴ and *in vivo*¹⁵. By effectively invading host cells, *P. gingivalis* can escape host immunity. Effective invasion of host cells not only

allows the bacterium to escape host defenses but also serves as a reservoir for future re-infection as well as alters the host cell. Studies of the molecular mechanisms involved in adhesion and internalization of the bacterium by host cells are needed. Research in several laboratories is focused on understanding the molecular events associated with internalization of *P. gingivalis* by the host cells as well as the mechanisms used to suppress and hijack the immune response and survive the hostile host defense mechanisms.

There are many assays capable of identifying and characterizing pathogens that are capable of invading host cells. However, *in vitro* studies with anaerobic pathogens pose many experimental problems for the researcher mainly because it is difficult to perform studies that rely on bulky instruments in the absence of oxygen. This is compounded by the fact that eukaryotic cells require oxygen to grow and thus must be prepared separately in tissue culture incubators. One way to avoid such obstacles would be to perform the studies under atmospheric oxygen, but that would make growth of anaerobic bacteria impossible. Another method would be to use heat-killed bacteria to infect and study host-cell interactions. However, differences exist between heat-killed and viable bacteria that diminish the relevancy of the host-pathogen interaction¹⁶. It is central to study viable bacteria with unaltered expression interacting with host cells; therefore, methods for culturing *P. gingivalis* in an anaerobic setting are given. Also, two simple cost-effective protocols are demonstrated for assessing the ability of *P. gingivalis* to be internalized by human umbilical vein endothelial cells (HUVECs). The first protocol is based on the popular antibiotic protection assay. Although the assay is straightforward, considerations when using anaerobic microorganisms are given. The second protocol requires the use of a fluorescent scanning microscope to visualize interacting and internalized *P. gingivalis*. Each assay has its limitations and advantages that will be discussed to provide the researcher an outline for studying the invasiveness of anaerobic bacteria. Although the current manuscript studies *P. gingivalis* and HUVECs, these protocols can be used for many other anaerobic bacteria as well as for other types of host cells.

Protocol

The following protocols will describe methods for culturing and studying the invasion by the anaerobic species, *P. gingivalis*; however, these protocols may be used for a number of anaerobic pathogens. Although HUVECs are used, this protocol may be used for other eukaryotic cells both immune and non-immune.

1. Anaerobic Chamber Use and Maintenance

Note: *P. gingivalis* is an anaerobe sensitive to normal levels of oxygen encountered in ambient air. A controlled anaerobic environment is vital for the cultivation of *P. gingivalis*.

1. Here, maintain an artificial atmosphere designated as mixed anaerobic gas (80% N₂, 10% H₂, 10% CO₂) in a vinyl anaerobic chamber (**Figure 1A**). Use an airlock (**Figure 1B**) for transferring items from the laboratory environment to the anaerobic chamber. The airlock operates manually, twice purging with N₂ gas before introducing the mixed anaerobic gas.
2. Use a hydrogen sulfide removal column (**Figure 1C**) for maintenance-free removal of the undesirable hydrogen sulfide. Place a dehumidifier within the chamber to remove H₂O created by the catalyst and to avoid aerosols that facilitate the spread of contamination.
Note: Hydrogen sulfide is a natural metabolic byproduct of many anaerobic bacteria and its accumulation is toxic to bacteria and can result in damage to electronics and decrease the lifetime of a catalyst.
3. Use a fan box to circulate the chamber's atmosphere through a palladium catalyst, which removes oxygen in the presence of hydrogen (**Figure 1D**).
Note: A recirculating atmospheric (HEPA) filter removes airborne contaminants with a size of 0.22 µm or larger.
4. Culture anaerobic bacteria in a 37 °C incubator that is located inside of the anaerobic chamber. Use standard aseptic techniques when working inside the anaerobic chamber.

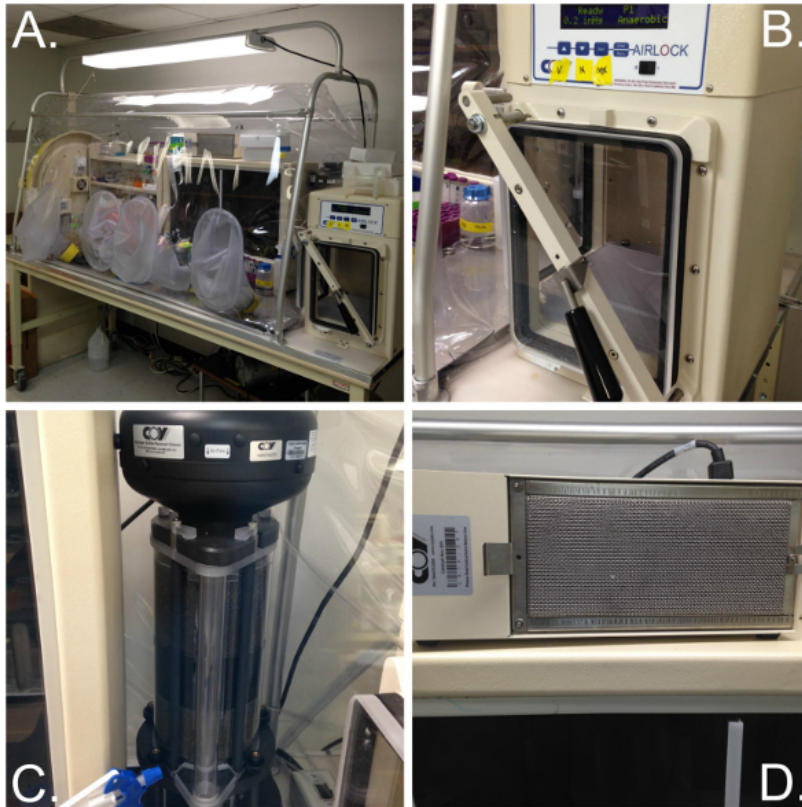


Figure 1. Anaerobic vinyl chamber and its components. (A) A vinyl anaerobic chamber sealed completely from atmospheric oxygen provides workspace for two individuals at a time (32 in x 78 in). It contains an incubator set at 37 °C (back middle). (B) An airlock is used for the transfer of items from the lab environment to the anaerobic chamber. Pictured is an automatic airlock operated through a controller that can be programmed to automatically perform the vacuum and purge procedures needed to create an anaerobic environment. (C) A Hydrogen Sulfide Removal Column provides maintenance-free high capacity removal of undesirable hydrogen sulfide. (D) Two catalyst fan boxes are placed throughout the anaerobic chamber to help circulate the chamber's atmosphere through palladium catalyst, which, in the presence of hydrogen, removes oxygen. The anaerobic chamber is set up according to manufacturer's instructions. [Please click here to view a larger version of this figure.](#)

2. Preparation of Anaerobic Bacteria

Note: *P. gingivalis* is aerotolerant and can be stored in aerobic conditions but it will not grow in the presence of oxygen at levels higher than 6%^{17,18}. An anaerobic chamber is necessary for the proper cultivation of *P. gingivalis* and other anaerobic species (Figure 1). Proper training and education on anaerobic chamber use is required before working with microanaerobes¹⁹.

1. Equilibrate all liquid media and plates to anaerobic conditions for at least 12 hr prior to experimentation to remove residual oxygen.
2. Transfer *P. gingivalis* from -80 °C freezer to anaerobic chamber, let thaw.
3. Streak *P. gingivalis* on trypticase soy blood agar plates (TSA II with 5% sheep blood). Wrap plates in parafilm and store at 37 °C in an anaerobic incubator for 4-7 days.
4. Inoculate *P. gingivalis* into 3 ml brain heart infusion (BHI) broth supplemented with hemin and menadione, an enriched non-selective liquid media for the isolation and culture of anaerobic and fastidious microorganisms, using sterile loops.
Note: For long-term storage, mix bacterial cultures prepared in BHI with glycerol or DMSO (10-20% final concentration) and place in a -80 °C freezer.
5. Prepare a starter culture of *P. gingivalis* by making a 1:10 dilution and allowing bacteria to grow until mid-log phase.

Note: The optical density of the bacterial suspension is determined and the bacterial concentration for each strain to be examined is adjusted. For *P. gingivalis* a suspension at OD₆₆₀ of 0.7 corresponds to mid-log phase and $\sim 7 \times 10^8$ cells/ml. Growth conditions described in the protocol above are specific for *P. gingivalis* and may need to be adapted for other bacterial strains.

3. Endothelial Cell Culture

Note: Purchase pooled primary HUVECs and culture in basal medium containing vascular endothelial growth factors (VEGF) at 37 °C in 5% CO₂ according to manufacturer's instructions.

1. Seed HUVECs in T-75 flasks at 2.5×10^5 cells/flask in 15 ml VEGF media.

Note: Check viability via a 1:1 dilution with 4.0% trypan blue. Cells with a compromised membrane will retain trypan blue, healthy cells with intact membranes will appear white when viewed under a binocular light microscope. Count 100 cells, ensure that over 80% of the cells are viable²⁰.

2. Replace media every 2 days with pre-warmed fresh VEGF media until cells reach ~80% confluency.
3. Wash cells once with pre-warmed PBS. Liberate cells from the T75 flask by incubating with 2 ml trypsin-EDTA (0.25%) for 5 min followed by 2 ml trypsin neutralizing solution.
4. Collect suspended HUVECs in a 50 ml conical tube. Wash out any extra cells from T-75 flasks with PBS and transfer to 50 ml conical tubes.
5. Centrifuge cells at 200 x g for 10 min.
6. Remove supernatant, suspend cell pellet in 10 ml pre-warmed VEGF media.
7. Determine cell concentration using a hemocytometer or similar cell counting device.
8. Calculate amount of cell suspension to add to either 6-well plate (400,000/well) or 12-well plate with coverslips (50,000/well). HUVECs will be ready for experimentation the next day.

4. Survival Assay Invasion/Interaction (Plating)

Note: When performing this assay, prepare two 6-well plates of endothelial cells seeded at 400,000 cells/well. One plate will be used to assess bacteria attached to and internalized by host cells. The other plate will account for intracellular bacteria. The 6-well plate allows for triplicates of two samples to be performed in one experiment. For an outline of this protocol please refer to the survival assay flowchart (**Figure 2**).

1. Prepare anaerobic bacteria as described above (see section 1) until they reach mid-log growth (OD_{660} 0.5-0.7).
2. Centrifuge bacteria at 5,000 x g for 10 min.
Note: If centrifuge is outside anaerobic chamber, carry bacterial samples in a tightly sealed 15 ml tube, wrap the cap with parafilm to prevent oxygen leakage.
3. Place pelleted *P. gingivalis* back in chamber, discard supernatant. Wash with PBS, pellet bacteria again before resuspending in VEGF media. Prepare suspensions for all bacterial strains to be tested at OD_{660} of 0.7 which corresponds to mid-log phase ($\sim 7 \times 10^8$ cells/ml). The bacteria are now ready for infection.
4. Transfer 6-well plates containing HUVECs from tissue culture incubator into anaerobic chamber. Remove media and wash three times with anaerobic PBS. Add 2 ml of anaerobic VEGF media to each well and place the plates at 37 °C in the anaerobic incubator for 20 min to equilibrate the temperature for infection.
Note: Plate bacteria on blood agar plates to ensure the ones used for infection are homogenous and not contaminated upon infection.
5. Infect host cells with bacteria at a multiplicity of infection (MOI bacteria: host) of 100:1.
Note: HUVEC cell number is determined by performing a trypan exclusion test on a single well before infection. Bacterial cell number is determined via optical density (e.g., OD of 0.5 = 5×10^8 cells/ml). Bacterial concentration is adjusted to proper MOI based on HUVECs concentration²¹.
6. Place 6-well plates with infected HUVECs into anaerobic incubator and allow bacteria to interact with host cells for 30 min.
7. Prepare saponin in BHI (1.0% w/v) inside the anaerobic chamber and filter through a 0.2 μ m filter.
8. Survival of both attached and internalized bacteria.
 1. Remove plates from the incubator, aspirate media, wash three times with anaerobic PBS and add 2 ml filtered 1.0% saponin (prepared as described in step 4.8). Incubate for 15 min to allow host cell lysis.
 2. Scrape bottom of each well with cell scraper. Collect the cell mixture from each well and make a 1:1 dilution in BHI.
 3. Proceed to make serial dilutions of the sample. Depending on bacterial species and concentration, adjust serial dilutions. Start with 1:100 or 1:1,000 dilutions.
 4. Plate 200 μ l of desired dilution on blood agar plates. Wrap the plates in parafilm and place in the anaerobic incubator at 37 °C.
 5. Following seven days of incubation at 37 °C, remove the plates and count colony forming units (CFUs) using a light box to manually count colonies.
Note: CFUs are enumerated. For larger quantities of CFUs, images may be taken and computer software can be used to facilitate the enumeration of CFUs.
9. Survival of internalized bacteria.
 1. Remove plates from the incubator. Wash three times with anaerobic PBS and add 2 ml VEGF media with supplemented antibiotic (300 μ g/ml of gentamicin and 400 μ g/ml of metronidazole).
 2. Incubate for 1 hr. Be sure to test the antibiotics so they are 100% effective at killing the desired bacterial strain and make sure that they do not penetrate host cells^{22,23}.
 3. Aspirate media, add 2 ml of filtered 1.0% saponin. Incubate for 15 min to allow host cell lysis.
 4. Scrape bottom of each well with cell scraper. Collect the cell mixture from each well and make 1:1 dilution in BHI.
 5. Prepare serial dilutions of the sample (1:100, 1:1,000).
 6. Plate 200 μ l of desired dilution on blood agar plates. Wrap plates in parafilm and place in anaerobic incubator.
 7. After seven days of incubation at 37 °C remove plates and count CFUs.

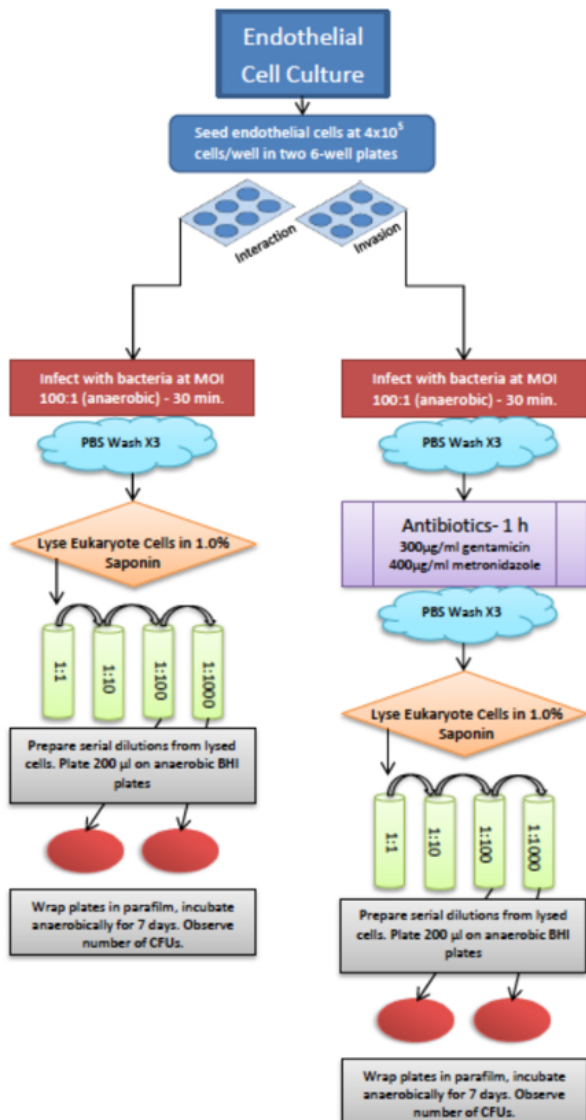


Figure 2. Schematic representation of a protocol used for survival of anaerobic bacteria with eukaryotic cells. Both assays for a total bacterial survival and survival of internalized bacteria can be performed at the same time. [Please click here to view a larger version of this figure.](#)

5. Internalization of Bacteria into Host Cells (Fluorescent Microscopy)

Note: *P. gingivalis* is labeled with 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM). BCECF-AM is a non-fluorescent membrane-permeable dye; its conversion to fluorescein BCECF via the action of intracellular esterases can indicate cell viability. *P. gingivalis* is labeled with the BCECF-AM dye and then used to infect eukaryotic cells. Following infection, cells are fixed and labeled with DAPI and TRITC-phalloidin. The DAPI stain used to stain the eukaryotic cell nucleus will also label bacterial cell nucleus, which provides a counter-measure to identify non-viable bacteria that cannot metabolically cleave BCECF-AM. Host cells are highlighted with TRITC-phalloidin, a red actin dye.

1. Autoclave coverslips. Aseptically add coverslips to 12-well plates before seeding endothelial cells at 5×10^4 cells/well. (Prepared day before experiment)
2. Have endothelial cells prepared on 18 mm (#1.5 thickness) circular coverslips in 12-well plates as described above.
3. Prepare anaerobic bacteria grown to mid-log phase ($OD_{600} = 0.5-0.7$) as described in section 1.
4. Wash bacteria 2x with anaerobic PBS by centrifuging at $5,000 \times g$ and suspending pellet in PBS at $5-7 \times 10^8$ cells/ml.
5. Add 20 µl of 0.2 mM BCECF-AM to 2 ml of bacterial suspension ($5-7 \times 10^8$ cells/ml) to a final concentration of BCECF-AM of 2 µM.
6. Incubate at 37 °C for 30 min in the dark.
7. Transfer plates with endothelial cells seeded on 18 mm (#1.5 thickness) circular coverslips from tissue culture incubator into the anaerobic chamber. Wash with PBS and exchange with anaerobic VEGF media.
Note: Verify that HUVECs are healthy under a light microscope. HUVECS should be ~80% confluent, morphology should be comparable to the manufacturers.
8. Centrifuge labeled bacteria at $5,000 \times g$ for 10 min to remove residual BCECF-AM dye. Suspend in 2 ml anaerobic VEGF media.
9. Infect host cells with labeled bacteria at MOI of 100:1 (bacteria: host).

10. Incubate in anaerobic chamber at 37 °C for 30 min.
11. After infection wash cells with PBS three times and fix in freshly prepared 4.0% paraformaldehyde for 10 min.
Note: After fixing cells, experiment can be conducted outside of the anaerobic chamber.
12. Wash coverslips with PBS three times.
13. Add 1 ml of 0.2% Triton X-100 for 10 min.
14. Wash coverslips with PBS three times.
15. Add 50 µl of TRITC phalloidin (50 µg/ml) to coverslips for 45 min.
16. Wash coverslips three times, remove from the 12-well plate and place on a slide with soft-set mounting medium containing DAPI. Seal the sides with nail polish.
Note: Slides may be stored for a couple months in the dark. Avoid light exposure to prevent photo-bleaching.
17. View slides using a confocal microscope.
 1. Here, use a 34 channel spectral system (32-channel array detector and two side PMT detectors, plus a transmitted light detector) configured around an AxioObserver (inverted) stand with a motorized XY stage. The system has five lasers: blue diode (405 nm), multi-line Argon (458, 488, 514 nm), green diode (561 nm), red HeNe (633 nm) and a 440 nm pulsed laser. Equip a Fluorescence Lifetime Imaging system with 2 hybrid GaAsP detectors (for FRET-FLIM).
 2. Detect fluorescence from DAPI and TRITC in one channel using a dual-band filter with excitation wavelengths of 340-380 nm and 540-560 nm, and an emission filter of 435-485 nm and 570-590 nm respectively. Detect fluorescence from BCECF-AM using a filter with excitation wavelength of 440-500 nm and an emission filter of 510-590 nm.
Note: Controls for BCECF-AM should be done on every bacterial strain being studied to ensure proper labeling of viable bacteria. First validate that nonviable bacteria are DAPI-positive and BCECF-negative. Second, ensure that live bacteria can metabolize BCECF-AM into fluorescein BCECF. Variable concentrations of the bacteria or BCECF-AM dye may need to be tested for optimal labeling.

Representative Results

Protocols outlined above were used in studying host-pathogen interaction between *P. gingivalis* and endothelial cells. *P. gingivalis* W83 and a *P. gingivalis* V3150 carrying a deletion of PG0228 were used in the study. The PG0228 is predicted to encode a protein that may alter the levels of RNA and proteins, which may ultimately affect interaction of *P. gingivalis* with host cells. To investigate the effect of PG0228 on *P. gingivalis*'s ability to interact with host cells, the ability of the parental and mutant strains to interact with HUVECs was compared. The survival protocol (**Figure 2**) was applied to this study. Thus, both strains were grown to logarithmic phase as outlined in step 2. Both strains had similar growth curves and thus no significant problems with normalization of the cell numbers prior to infection was encountered. Following seven days of incubation, CFUs were observed on the blood agar plates (**Figure 3**). Several dilutions 1:10 (**Figure 3A**), 1:100 (**Figure 3C**) and 1:1,000 (**Figure 3B**) were plated on blood agar plates. As seen in **Figure 3A** the number of colonies were too high to assess; the colonies were indiscernible from each other and designated as "too many to count" [TMTC]. The dilution in **Figure 3B** was too low as too few colonies are obtained. The dilutions of 1:100 as shown in **Figure 3C** was desirable, as the colonies are discernible and the number of colonies were manageable to count. Similar results were found for the mutant strain V3150 (**Figure 3D**). Thus the CFUs were enumerated from the plates with the dilution factor of 1:100.

Using the number of CFUs and the dilution factor, the estimated number of viable bacteria recovered for each strain was calculated. Dividing the number of viable bacteria recovered by the initial number of bacteria results in a ratio defined as the invasion efficiency. In comparing the invasion efficiencies of both strains, wild type W83 survived within HUVECs with high efficiency, whilst strains lacking PG0228 showed a significant reduction in survival (**Figure 4**).

To further verify that the defect was due to survival rather than reduced internalization of the bacteria, microscopy analysis was performed. HUVECs infected with *P. gingivalis* were viewed under a microscope (**Figure 5**). In this example, one HUVEC is presented. To determine the number of internalized bacteria multiple images were taken at different focal distances to obtain a Z-stack allowing for spatial-temporal identification of internalized *P. gingivalis*. Also, at least 40 cells/experiment should be analyzed (for each biological replicate) and the number of internalized bacteria enumerated for each strain. If the number of internalized bacteria is the same for each strain when viewed under a microscope then both strains invade HUVECs equally; however, the mutant V3150 has reduced ability to survive while within the host cell as seen in the antibiotic protection assay.

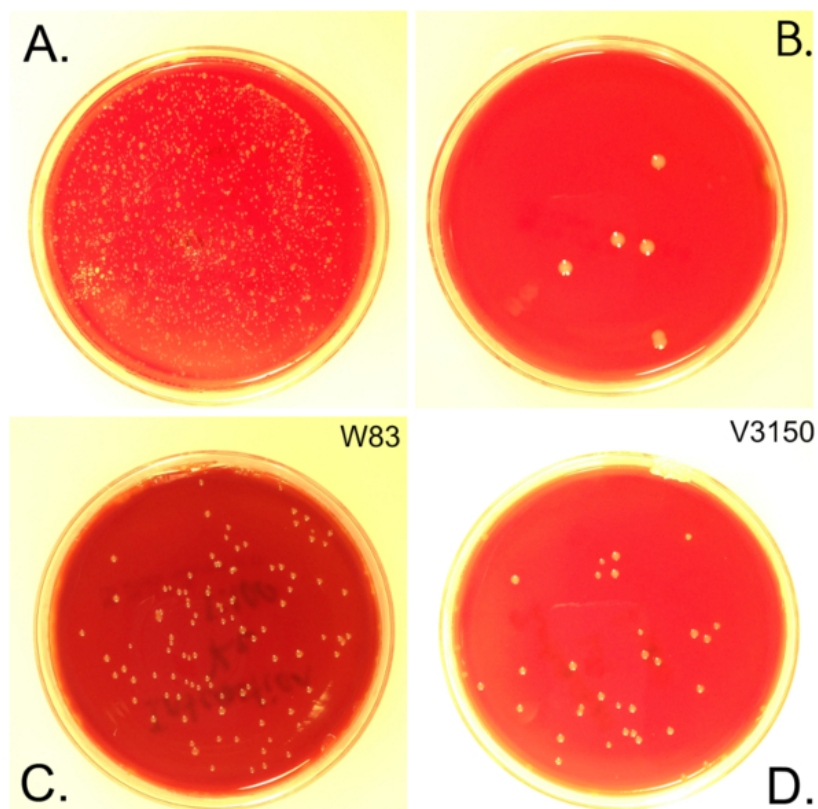


Figure 3. Results from a bacterial survival assay. Protocol for survival of intracellular bacteria was used to assess the ability of wild type *P. gingivalis* W83 and a mutant V3150 deficient in PG0228 to invade and survive within HUVECs. Internalized viable bacteria are determined by visualization of CFUs following incubation of HUVECs-*P. gingivalis* mixture on blood agar plates for seven days under anaerobic conditions. Blood plates are placed on light box that increases contrast and allows for easier counting of the CFUs. Various dilutions were examined. HUVECs infected with *P. gingivalis* W83 dilution 1:10 (A), dilution 1:1,000 (B), and dilution 1:100 (C). HUVECs infected with *P. gingivalis* V3150 dilution 1:100 (D). [Please click here to view a larger version of this figure.](#)

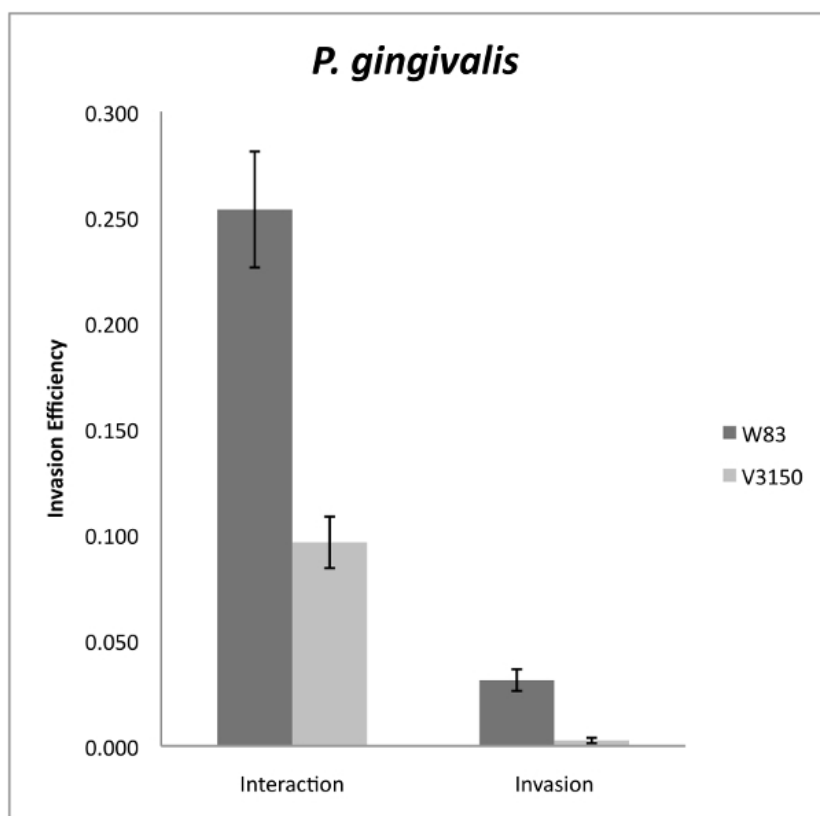


Figure 4. Comparison of a survival rates between parental and mutant bacterial strains. HUVECs were infected with wild type *P. gingivalis* W83 and a mutant V3150 deficient in PG0228. Protocol for survival as outlined in **Figure 2** was followed. Experiment was performed three times in triplicates and the mean $\pm$ standard deviations are presented. Invasion efficiency represents survived/initial bacteria. *The mutant strain has statistically significant reduced survival when compared to the wild type W83 strain ($P < 0.05$). [Please click here to view a larger version of this figure.](#)

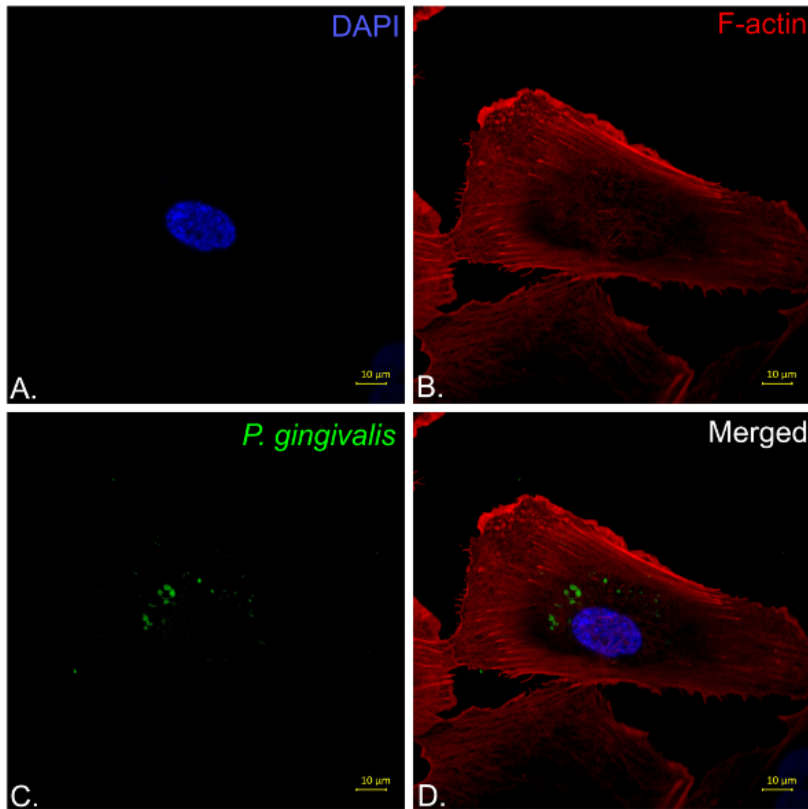


Figure 5. Example of a microscopic analysis of *P. gingivalis* internalized by HUVECs. HUVECs were infected with BCECF-AM labeled *P. gingivalis* for 30 min at an MOI of 100:1 (*P. gingivalis*: HUVEC). Cells were fixed with paraformaldehyde and labeled with TRITC-phalloidin and DAPI. The image shows a single HUVEC with the nucleus (A) and F-actin (B) stained blue and red, respectively. The arrow indicates *P. gingivalis* labeled green (C). A merged image is produced to show *P. gingivalis* invasion into HUVECs (D). Images were produced using scanning confocal microscope. [Please click here to view a larger version of this figure.](#)

Discussion

All the above methods can be used to design specific assays to assess the interaction of anaerobic bacteria with eukaryotic cells. However, there are several considerations to successfully perform the experiments. First are the microbial strains to be used in a study.

It is crucial in the comparison of two strains with both the survival assay as well as by microscopy analysis that they are at similar growth phases and attain similar cell concentrations as any differences in the above can influence invasion efficiency¹³. When growth curves differ between two bacterial strains, adjustments should be made to ultimately attain similar growth patterns sharing consistent concentrations²¹. Also, bacteria should be dispersed evenly to avoid cell aggregation. Furthermore, it is critical to select antibiotics that effectively eliminate extracellular bacteria. If chosen antibiotic has deleterious effects on host cells, then an alternative antibiotic or lytic enzymes may be used^{24,25}. Finally, strain heterogeneity must be considered; although a species may be identified to invade host cells, there can be major difference in pathogenicity from one strain to another²⁶ and thus a pilot study should be performed for each strain to be examined.

A second critical step is to establish an optimal incubation time and bacterial inoculum (multiplicity of infection [MOI] which is the number of bacteria used to infect host cell) when designing the protocols. Shorter incubation periods will assess the ability of bacteria to be internalized by host cells while longer time points may be needed to determine bacterial survival as well as intracellular replication²⁷. As intracellular survival of bacteria could be affected by the MOI being used, it is best to test multiple MOIs to ensure that no damage to cell was caused that would lead to cell death or permeabilized membranes with access to antibiotic killing of intracellular bacteria. After establishing MOI and incubation times needed to answer the specific question being asked, the experiment is run according to the protocol; however, several serial dilutions of the eukaryotic cell-bacteria lysis mixture should be made. The optimal dilution factor will be determined based on CFUs observed. Dilution factors that result in plates with 50-200 CFUs are optimal for assessing survival efficiency. If the CFU count is too high, colonies are indiscernible from each other and it becomes difficult to manually count each colony. If the CFU count is too low, small deviations exaggerate the fold change between strains being examined as well as produce large standard deviations between replicates.

A third critical point is to prepare host cells that are healthy and ready to interact with bacteria. In the above protocol host cells are cultured separately using standard aseptic techniques. The use of antibiotics and antifungals in tissue culturing may be advisable, especially for beginner cell culture biologists. However, before performing the survival assay, host cells should be cultured in antibiotic-free media for at least 12 hr before transferring the cells into the anaerobic chamber. Once inside the chamber, media should be exchanged with anaerobic antibiotic-free media as to not impinge on anaerobic bacteria during infection. If problems exist with attachment of host cells to plastic tissue culture plates or coverslips it may be advisable to apply an adhesive coating such as poly-L-lysine or gelatin. Also, techniques for coating tissue culture dishes with a naturally produced basement membrane-like extracellular matrix (ECM) may provide the investigator with more *in vivo* like conditions^{28,29}.

Lysing host cells requires a balanced detergent capable of opening up host cells without altering bacterial viability. Although saponin will not kill bacteria, it may inhibit growth of some species. Prior to our studies the effect of saponin on *P. gingivalis* strains to be used for the host-pathogen analysis was verified to have no effect on their growth/survival. If bacteria are highly sensitive to detergents, including saponin, repeated freeze-thaw cycles or distilled water may be used to lyse host cells with little damage to bacteria.

A fourth critical point includes the considerations to be taken when performing the microscopy experiments. First is the use of a fluorescent dye to label bacteria to be used for the microscopy protocol. Many current labeling methods for bacteria require a primary antibody or customary bacterial dyes with significant limitations, such as toxic effects and leaching of the dye into surroundings thus giving high background. BCECF-AM is a membrane-permeable dye commonly used as a fluorescent indicator of intracellular pH in both prokaryote and eukaryotes³⁰⁻³². It was found to be effective at labeling a range of anaerobic bacteria in previous studies³³⁻³⁵. BCECF-AM will only label viable bacteria capable of esterification. The conversion to BCECF by intracellular esterases results in a fluorescent form that leaks out of cells far more slowly than its parent compound. There are many fluorescent dyes and staining techniques that may be used³⁶; however, this protocol outlines a simple fixing/staining technique that can be used with almost any microorganism. In addition, other dyes (TRITC-phalloidin, and DAPI) are used to more clearly distinguish the boundaries of the eukaryotic cells and account for only bacteria that interact with host cells.

Fluorescent dyes are susceptible to photo-bleaching and there are several commercial antifades and mounting mediums available that may prevent weakening of the fluorescent signal³⁷. There are also choices between hard-set mounting media and soft-set ones. While the hard-set ones may result in significant changes in the refractory index as well as shrinkage and tissue damage³⁸ the soft-setting media require sealants, such as nail polish, to secure the coverslip. Then due to variations observed amongst infected eukaryotic cells; the number of internalized bacteria between cells may vary and thus multiple eukaryotic cells should be used for analysis. In our studies, at least forty eukaryotic cells are assessed and internalized bacteria enumerated per experiment^{33,34}. Finally, to clearly visualize single cells, eukaryotic cells used for infection should not be grown to confluence. Microscopic examinations of *Prevotella intermedia* infection with epithelial cells showed preference for specific regions of the host cell membrane, and the bacteria would not adhere to sites where epithelial cells were in contact with each other and had no lamellipodia³⁹.

The limitation of the microscopy could be its low throughput. Thus, for large-scale quantitative data on the invasive property of bacteria a flow cytometry can also be used⁴⁰. This method involves the use of fluorescently labeled bacteria (that can be accomplished as described for bacteria to be used for microscopy) and allows for studying multiple samples at a time that may be attractive for some researchers.

Modifications to the two protocols can be made to identify pathways involved in host cell uptake of bacteria. Successful bacterial invasion occurs in five stages: (1) attachment (2) entry/internalization (3) trafficking (4) persistence and (5) exit⁴¹. Thus during entry/internalization, the bacterium locates itself upon the host and usurps the host cell for internalization through modifying signal transduction. Selective metabolic inhibitors can be added to host cells to determine changes in signaling leading to successful invasion. For example latrunculin, which inhibits actin polymerization, can be used to study how cytoskeleton rearrangement affects internalization of *P. gingivalis*. Antibodies that target cell-specific receptors or siRNA capable of knocking down certain genes may also be used for a better understanding of host cell signaling events involved in bacterial internalization. While all metabolic inhibitors should have minimal overall effect on host cells, except the one being investigated, it is important to ensure the inhibitor treatments do not have a toxic effect on bacteria.

Future studies would look to perfect some of these techniques, possibly attain a more *in vivo* like condition. In the current article, either bacteria or host cells are exposed to harsh environments. Depending on the organism, the cell line, and goal of the experiment some investigators may prefer to perform the above protocol in a CO₂ incubator. However, periodontal lesions reflect an anaerobic microenvironment in which the bacteria interact with the host. A study that examined variations in cellular response to challenge with oral bacteria under aerobic versus anaerobic conditions reported that under reduced oxygen tension (2% oxygen) bacteria, e.g., *Tannerella forsythia*, *P. gingivalis*, and *P. intermedia* elicited higher levels of IL-8 and TNF α compared to aerobic conditions⁴². The ability of eukaryotic cells to survive under anaerobic conditions was also confirmed by studies that examined the ability of human mesenchymal stem cells, human dental follicle stem cells, human gingival fibroblasts, gingival carcinoma cells, and human oral epithelial cells^{43,44,45}. Our studies using WST-1 cell proliferation assay showed that HUVECs can survive for up to 48 hr under anoxic conditions. The decision was made to infect the cells in the anaerobic chamber because *P. gingivalis* is sensitive to atmospheric oxygen levels, while HUVECs can survive extended periods of time under anaerobic conditions. Future research will investigate implementation of micro-fluidic cell culture devices that provide oxygen to one surface of a monolayer (like an artery) and anaerobic conditions at the other⁴⁶. This way conditions will not be sacrificed for either bacteria or host upon infection. In summary, described are a few simple protocols that can be used to examine host-pathogen interaction for anaerobic bacteria. These protocols have also been used to assess the effect of bacterial internalins, proteins promoting bacterial invasion⁴⁰, as well as surrogate non-invasive bacteria expressing a protein conferring invasive property on the bacteria⁴⁷.

Disclosures

Authors have nothing to disclose.

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