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Flowcell culture of *Porphyromonas gingivalis* biofilms under anaerobic conditions

Martin C. Hansen[a], Robert J. Palmer Jr.[b,*], David C. White[b,c]

[a]*Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark*
[b]*Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932, USA*
[c]*Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA*

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Abstract

We have developed an anaerobic biofilm culture system. The system is inexpensive, simple to use and, unlike an anaerobic glovebox, requires no dedicated space. As a test of the system, *Porphyromonas gingivalis* was cultured under low oxygen (1–2 ppm) and under anaerobic conditions (≤ 0.1 ppm O_2). In the presence of small amounts of oxygen, the organism attached and formed an initial biofilm over the course of 4 h, but the biofilm was unable to maintain its growth and had lost biomass after 18 h. Also, ambiguous results were obtained when the biofilm was stained with a viability stain. Under anaerobic conditions, the biofilm was able to continue growth — biomass was greater after 18 h than after 4 h, and the anaerobic biofilm had a less ambiguous staining pattern than did the low-O_2-grown biofilm.

Keywords: Biofilm; Anaerobic; Laser confocal microscopy; *Porphyromonas gingivalis*; Plaque; Periodontal disease

1. Introduction

Oral biofilms such as dental plaque contain a wide variety of bacterial phenotypes, particularly with respect to oxygen sensitivity (Marsh and Martin, 1992). Some cells, such as most oral streptococci, are facultative anaerobes and are capable of growth under aerobic and anaerobic conditions. Other species, such as *Actinobacillus actinomycetemcomitans*, are capnophilic (CO_2-loving) and require, or grow much better in the presence of, elevated CO_2 (typically 5%); they do not however require anaerobic conditions. Still others, e.g., *Porphyromonas gingivalis*, are true anaerobes; these grow only in the absence of oxygen. The vast majority of oral anaerobes are oxygen-tolerant, meaning that they die slowly when exposed to oxygen (in contrast to, e.g., methanogens that die instantly upon exposure to air) and that routine subculture can be performed on the benchtop rather than in a glove box. This wide range of oxygen tolerance would be expected for bacteria isolated from the oral cavity: an environment that presents all the above-described conditions.

Flowcells (flow-through biofilm culture devices) are becoming the method-of-choice in aerobic

*Corresponding author. Current address: NIDCR/NIH, Bldg 30, Room 308, 30 Convent Drive, Bethesda, MD 20892, USA. Tel.: +1-301-594-0025; fax: +1-301-402-0396.
E-mail address: rjpalmer@dir.nidcr.nih.gov (R.J. Palmer Jr.)

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biofilm studies for reasons too extensive to elaborate upon here (e.g., Caldwell and Lawrence, 1988; Sjollema et al., 1990; Korber et al., 1994; Kolenbrander et al., 1999; Palmer, 1999; Zinn et al., 1999). Little work however has been performed on anaerobic biofilms, and the development of an anaerobic flowcell system would encourage such work. Occasionally, a flowcell has been attached to, e.g., an anaerobic bioreactor and the biofilm that developed in the flowcell was assumed to represent the community within the bioreactor (D. Korber, personal communication; A. Peacock and R.J. Palmer Jr., unpublished). However, little effort was made to examine conditions within the flowcell to determine whether those conditions approximated those within the bioreactor (i.e., anaerobic). Some completely anaerobic chemostat systems exist (e.g., Freter et al., 1983; Gibson et al., 1983; Bradshaw et al., 1996) but these have not been adapted specifically to in situ biofilm research. We have used a flowcell system for the culture of facultative anaerobes and for capnophilic organisms by simply sparging the medium reservoir with N_2:CO_2 (95:5) (Palmer, unpublished observations). We assumed conditions within the flowcell to be close to those within the medium reservoir (i.e. little to no oxygen, elevated CO_2). However, when we began to work with bacteria that express Green Fluorescent Protein (Hansen et al., submitted, Appl. Environ. Microbiol.), we discovered that the protein, which requires oxygen to become fluorescent (Heim et al., 1994), was brightly fluorescent under these conditions. Our ability to grow capnophiles demonstrated that the CO_2 concentration within the flowcell was higher than atmospheric, however the inability to generate non-fluorescent GFP demonstrated that oxygen was present. Thus, conditions within the flowcell did not reflect those within the medium reservoir.

We report here the adaptation of this flowcell system for use as an anaerobic biofilm culture setup. The setup is simple to use, inexpensive, requires little space, and thus has several advantages over a rigid glove box. We demonstrate, by a colorimetric assay and by the growth of an obligately anaerobic oral bacterium, that the oxygen level within the flowcell is very low. We also suggest that this system could be used to culture oxygen-intolerant bacteria.

2. Materials and methods

2.1. Bacterial strain and growth media

Porphyromonas gingivalis (strain 381, kindly provided by Howard Kuramitsu, Dept. Oral Biology, State Univ. NY, Buffalo, NY, USA) was routinely maintained at 34°C in *Porphyromonas* Broth [Todd Hewitt broth (Difco) supplemented with 1 g yeast extract, 5 mg hemin, and 1 mg menadione per liter] as batch cultures in anaerobic pressure tubes (Bellco). The medium was prepared using strict anaerobe methods (boiling, cooling while sparging with 95:5 N_2:CO_2 for 1 h prior to tubing, head-gas replacement and autoclaving). Resazurine (0.0001%) was used as an Eh indicator and visual check of reducing conditions. Growth medium for the biofilms was either a 10-fold dilution of the *Porphyromonas* Broth, or a 10-fold dilution of the *Porphyromonas* Broth supplemented with 0.5 g/l L-cysteine per liter as a reducing agent.

2.2. Flowcell culture of biofilms

Porphyromonas gingivalis was grown as a biofilm in glass flowcells for microscopy (Palmer and Caldwell, 1995; Palmer, 1999). These flowcells are the same length and width as a standard microscope slide; the dimensions of the internal tracks (two per flowcell) are 1 mm deep and 2 mm wide, resulting in a total volume per track of approximately 250 μl. Five hundred microliters of a diluted (A_{600} of 0.1) overnight broth culture were injected into the flowcell and flow (5.5 ml/h) was begun after a 20-min attachment period. In capnophilic (low O_2) experiments, the flowcell medium reservoir was sparged with 95:5 N_2:CO_2, however no other attempts were made to prevent O_2 entry into the system — as indicated in Section 1, this system is suitable for growth of the capnophilic bacterium *Actinobacillus actinomycetemcomitans*, but the system is not anaerobic. For anaerobic experiments, the flowcell system was enclosed in an Inflatable Glove Chamber (Model X-17-17, Instruments for Research and Industry, Cheltenham, PA, USA) hereafter called the glove bag. In the glove bag were the reservoir (500 ml Erlenmeyer flask with medium), all tubing from

the reservoir to the flowcells, the flowcells and at least 20 cm of tubing downstream from the flowcells. Outside the glove bag, the tubing led to a peristaltic pump and waste container. Tubing from a 95:5 N_2:CO_2 flask entered the glove bag through its narrow opening, and served as a source of continuous sparging gas for the reservoir (the overpressure of which maintained a positive N_2:CO_2 pressure in the glove bag), as well as for rapid introduction of large volumes of gas into the bag during inflation/deflation cycles at the beginning of the experiment (Fig. 1). Once an anaerobic environment was initially obtained in the glove bag (after four rounds of inflation/deflation), positive pressure allowed small items such as syringes to be inserted or removed from the glove bag without disruption of the anaerobic conditions. The entire experimental setup was housed in a 34°C constant temperature room.

2.3. Measurement of dissolved oxygen

The concentration of dissolved oxygen in the tubed anaerobic medium and in the flowcell medium was determined colorimetrically using CHEMets Dissolved Oxygen Ampoules (Kit K-7501 and Kit K-7512, CHEMetrics, Inc. Calverton, VA, USA). Measurements of oxygen in the anaerobic setup were performed in the glove bag. For measurements from the low O_2 setup, an aliquot of medium was withdrawn from the downstream side of the flowcell using a thrice-purged (N_2:CO_2, 95:5) gas-tight syringe. The needle was inserted into a rubber stopper

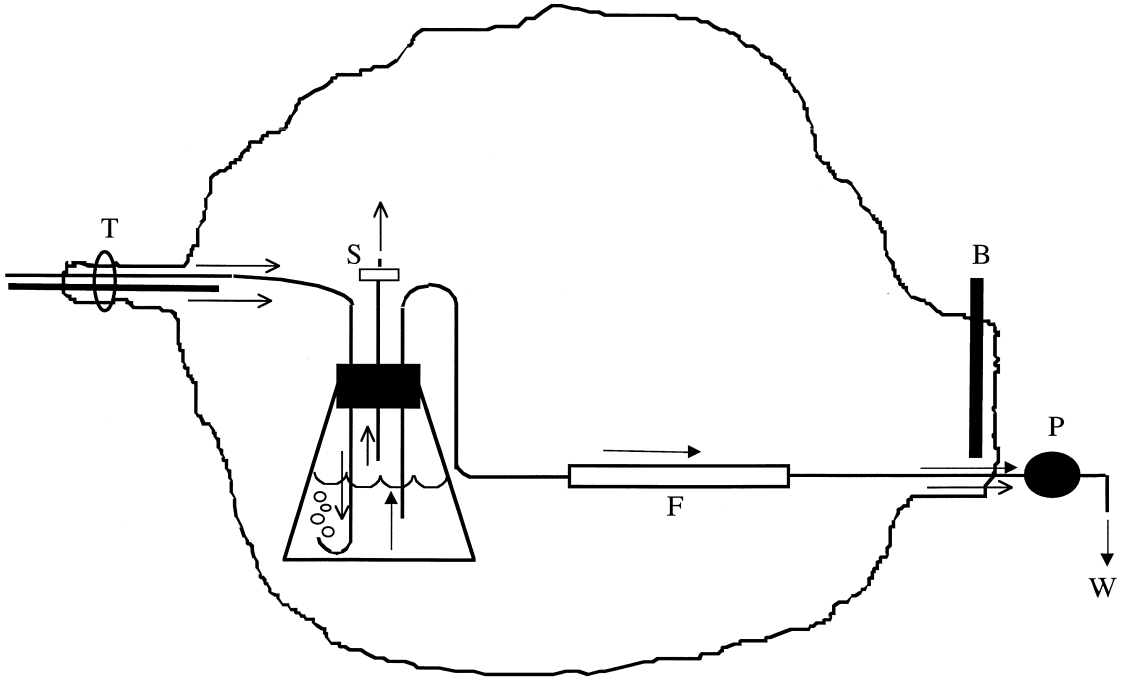

Fig. 1. Glove bag setup for anaerobic biofilm growth (not to scale). Gas flow shown with open arrowheads, medium flow shown with filled arrowheads. Two gas delivery tubes enter the bag from the left. Tape (T) is used to seal the bag entry tightly around the tubing. A small-diameter tube (Teflon or other gas-impermeable material) delivers filter-sterilized sparging gas to the medium reservoir. Overpressure exits the medium reservoir through a sterile filter (S), and is sufficient to keep the bag inflated if properly sealed. A large-diameter tube is used to fill the bag during initial inflation/deflation cycles (sparging the bag). A plastic bar clip (B) is provided by the bag manufacturer and is used to partially seal the large opening in the bag through which the flask and other components are introduced. The clip can be loosened during inflation/deflation and, once the bag interior is sparged, it is tightened to restrict gas exchange around the tubing exit. Sparged medium is pulled through the flowcell (F) by a peristaltic pump (P) located outside the bag, and sent to a waste container (W).

and the syringe was transported to an anaerobic glove box where the measurement was performed

2.4. Microscopy

Flowcells were removed from the glove bag prior to microscopic examination. The biofilm was stained by injection of 500 μl BacLight Live/Dead viability stain (Molecular Probes, Eugene OR) (1.5 μl of each dye in 3 ml medium) and observed using a Leica TCS-NT laser confocal microscope. The microscope was operated as follows: dual excitation (488 and 568 nm), dual emission (530/30 BP into channel 1 to record Syto 9 fluorescence and 650 LP into

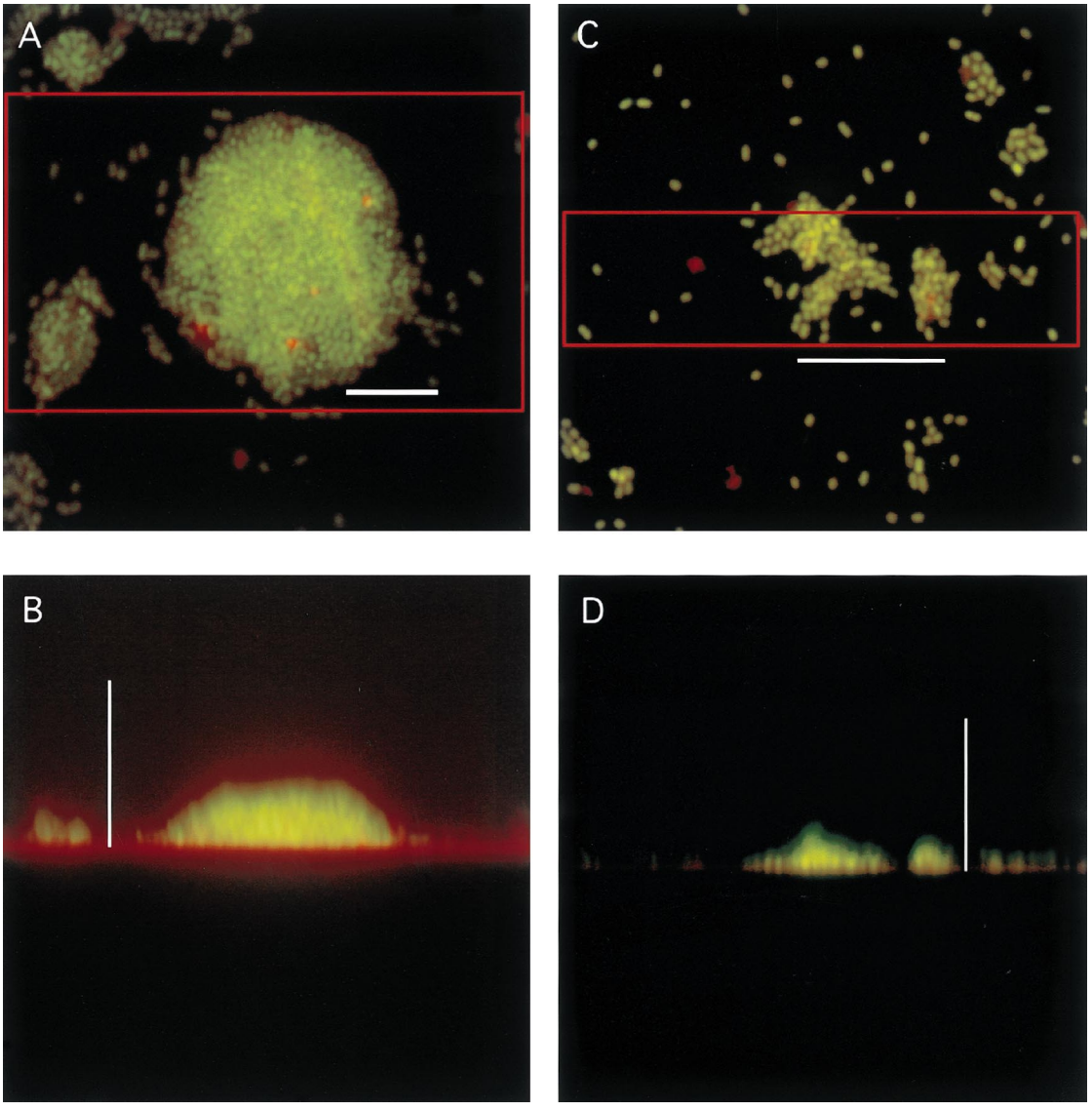

Fig. 2. *P. gingivalis* grown under capnophilic conditions. Biofilms stained with BacLight Live/Dead. (A,B): after 4 h of growth. (C,D): after 18 h of growth. Scale bars (white) = 10 μm. Red boxes in the ''top view'' images (A,C; *x*–*y* plane) demarcate the regions shown in cross-section (B, D; *x*–*z* plane). Images are maximum projections of the entire image stack with individual sections obtained at 0.5-μm intervals. See text for additional description.

channel 2 for propidium iodide fluorescence), 100× 1.4 NA oil immersion lens at an Airy disc setting of 0.9, 0.5-μm steps collected with four frames averaged at each step. Prior to imaging, PMT settings in both channels were adjusted interactively such that the RGB overlay (live scan) approximated the fluorescence observed through the oculars with a fluorescein LP filter (i.e. red, green, and yellow cells seen with the eyes were correlated to those imaged in the true-color overlay of the confocal scanner). The

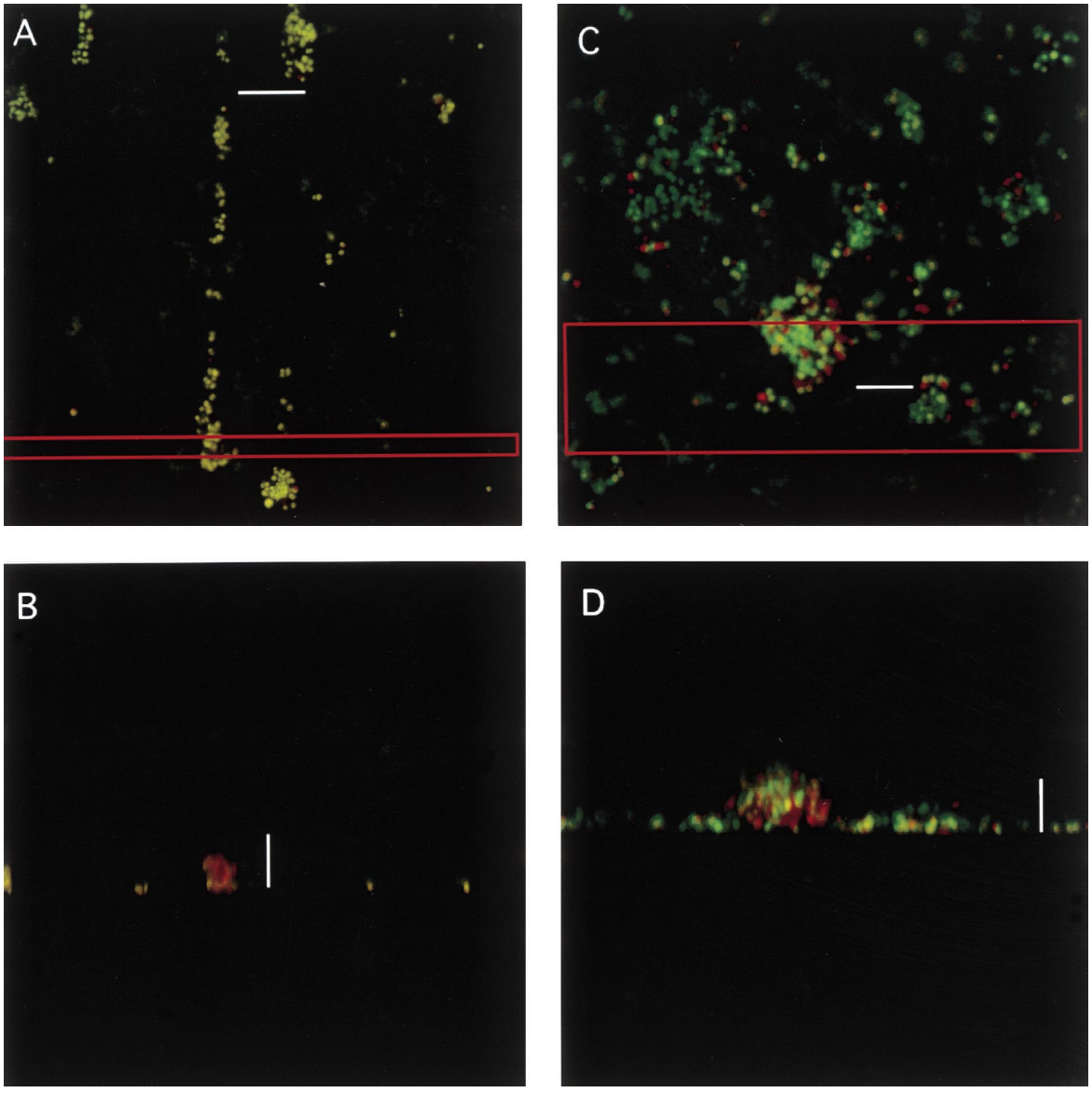

Fig. 3. *P. gingivalis* grown under anaerobic conditions. Biofilms stained with BacLight Live/Dead. (A,B): after 4 h of growth. (C,D): after 18 h of growth. Scale bars (white) = 10 μm. Red boxes in ''top view'' images (A,C; *x*–*y* plane) demarcate the regions shown in cross-section (B, D; *x*–*z* plane). Images are maximum projections of the entire image stack with individual sections obtained at 0.5-μm intervals. See text for additional description.

biofilms were subjected to microscopic examination after 4 h and after 18 h of growth (parallel experiments).

3. Results and discussion

Tubed anaerobic medium contained 0.1 ppm (0.1 mg/l) dissolved oxygen. This concentration might vary somewhat between medium batches as the trace amount of oxygen in the 95:5 N_2:CO_2 varies. The Eh indicator resazurine was colorless in the anaerobic pressure tubes and indicated that reducing conditions prevailed. Addition of L-cysteine to the medium as a reducing agent for the flowcells reduced the trace amount of dissolved oxygen to 0.025 ppm (assay sensitivity limit). By keeping the flowcells and the tubing inside the glove bag, an oxygen level of ≤ 0.1 ppm could be maintained. In the low-O_2 capnophilic setup, the oxygen level was 1–2 ppm.

In initial attempts to grow *P. gingivalis* biofilms, flowcells were not placed in the glove bag (i.e. were under low O_2 conditions). The medium reservoir was sparged and no attempts were made to reduce gas exchange through the tubing lines to the flowcell. Fig. 2 shows the biofilm obtained under these conditions. Cells attached and formed microcolonies during the 4 h of flow. Most cells were green to yellow (viable) although scattered red (non-viable) cells were present. After 18 h of development, the biofilm appeared to lose biomass — it was thinner (axial dimension) and more sparsely developed in the lateral directions than at the 4-h time point. Staining appeared more yellow than at the earlier time point. We interpret these images as suggesting that the cells were capable of attachment, but not of growth, under these conditions. Loss of attached cells occurred over time due to inability of the cells to grow and metabolize optimally. These data support in part the suggestion by others that *P. gingivalis* does not form biofilms under aerobic (or in our case, capnophilic microaerophilic) conditions (Cook et al., 1998). However, when our methods were adapted to provide strictly anaerobic conditions (≤ 0.1 ppm O_2), growth of the biofilm was markedly improved. Fig. 3 shows biofilm development under the anaerobic conditions afforded by the glove bag method. The cells are initially somewhat yellow (perhaps a reaction to the cysteine which was not normally included in the tube cultures from which the inoculum was taken) and a clear orientation with respect to flow (proceeds from top to bottom) was noticeable. The anaerobic biofilm was initially not significantly thicker than that obtained under microaerophilic conditions, however this could be inoculum-dependent. After 18 h of development, the strictly anaerobic biofilm showed clear growth over the 4 h timepoint. Staining had changed from yellow to primarily green with some scattered red cells. We interpreted these images as showing that growth of the biofilm proceeded under these conditions. Thus, when the cells were offered conditions suitable for growth, *P. gingivalis* did indeed form viable growing biofilms. To relate these data to occurrence of *P. gingivalis* in the oral cavity, the organism can be found in low frequency outside the sulcus, however it is unlikely to be able to grow under those environmental conditions. When in the more hospitable anaerobic environment of the gingival pocket, the organism can become an important component of a periodontal-disease-causing microbial community.

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