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Correlation Between Intraluminal Oxygen Gradient and Radial Partitioning of Intestinal Microbiota

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BACKGROUND & AIMS: The gut microbiota is a complex and densely populated community in a dynamic environment determined by host physiology. We investigated how intestinal oxygen levels affect the composition of the fecal and mucosally adherent microbiota. **METHODS:** We used the phosphorescence quenching method and a specially designed intraluminal oxygen probe to dynamically quantify gut luminal oxygen levels in mice. 16S ribosomal RNA gene sequencing was used to characterize the microbiota in intestines of mice exposed to hyperbaric oxygen, human rectal biopsy and mucosal swab samples, and paired human stool samples. **RESULTS:** Average Po_2 values in the lumen of the cecum were extremely low (<1 mm Hg). In altering oxygenation of mouse intestines, we observed that oxygen diffused from intestinal tissue and established a radial gradient that extended from the tissue interface into the lumen. Increasing tissue oxygenation with hyperbaric oxygen altered the composition of the gut microbiota in mice. In human beings, 16S ribosomal RNA gene analyses showed an increased proportion of oxygen-tolerant organisms of the *Proteobacteria* and *Actinobacteria* phyla associated with rectal mucosa, compared with feces. A consortium of asaccharolytic bacteria of the Firmicute and Bacteroidetes phyla, which primarily metabolize peptones and amino acids, was associated primarily with mucus. This could be owing to the presence of proteinaceous substrates provided by mucus and the shedding of the intestinal epithelium. **CONCLUSIONS:** In an analysis of intestinal microbiota of mice and human beings, we observed a radial gradient of microbes linked to the distribution of oxygen and nutrients provided by host tissue.

Keywords: Aerobic; Anaerobic; Microbe; Spatial Gradient of Oxygen.

The bacterial microbes that inhabit the intestinal tract form a complex community dominated by obligately anaerobic organisms from both the *Firmicutes* and *Bacteroidetes* phyla.[1] Carbohydrates are a major source of energy for the microbiota, which relies heavily on fermentative metabolism in the anaerobic environment. Considerable attention has focused on the mechanisms by which the saccharolytic microbes digest glycans to produce short-chain fatty acids that, in turn, influence host physiology.[2] Although the gut microbiota in human beings generally is stable in composition, alterations of the host that are induced, for example, by a change in diet[3,4] or intestinal inflammation,[5] can alter both the microbiota composition and function.[6] Within a healthy host, there are differences in the gut microbiota along the longitudinal axis of the gut,[7] as well as between different regions within a single fecal sample.[8] In this report, we examined the radial distribution of the gut microbiota and attempted to identify host-derived factors in the intestinal environment that could influence its composition. Identification of such factors could improve our understanding of the taxonomic differences between the mucosally associated and fecal microbiota in health as well as the development of the dysbiotic microbiota associated with intestinal inflammation.

The intestinal niche is largely devoid of oxygen. Several lines of evidence have implicated both aerobic and facultative anaerobic bacteria in the development of the anaerobic environment.[9] Culture-dependent surveys of the newborn microbiota suggest that the initial community of aerobic and facultative anaerobic bacteria might consume oxygen in the intestine, allowing the population of obligate anaerobes to develop.[10,11] This observation has been supported by more recent studies showing the predominance of oxygen-tolerant *Gammaproteobacteria* in the gut microbiota of newborns.[12] In addition, analysis of flatus composition in human beings shows a mixture of gases produced by microbial metabolism, namely hydrogen, carbon dioxide, methane, and hydrogen sulfide. Abundant levels of nitrogen also are present, but oxygen levels are very low.[13] However, direct evidence for the role of the gut microbiota in regulating oxygen levels in the intestinal lumen is still lacking.

The mechanism responsible for maintaining the anaerobic environment of the gut lumen is unclear, in part owing to the difficulty in quantifying intestinal oxygen levels.

***Authors share co-first authorship.**

Abbreviations used in this paper: **EPR, electron paramagnetic resonance; HBOT, hyperbaric oxygen therapy; PMMA, polymethylmethacrylate; rRNA, ribosomal RNA.**

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Although there have been several reports on oxygenation measurements of the intestinal tissue,[14,15] very few have described oxygen measurements directly in the lumen of the gut. Most experiments relied on Clark-type oxygen electrodes,[16–18] showing that the Po_2 in the lumen is less than 0.5 mm Hg.[16] However, use of electrodes is complicated by their invasiveness and potential leaks of oxygen into the measurement environment. Recently, electron paramagnetic resonance (EPR) oximetry was applied to image gut oxygen levels noninvasively in mice.[19] A spin probe (charcoal) was delivered orally, and the decay of the spin polarization was used to assess oxygen concentration.[20] The measured Po_2 levels decreased from 58 mm Hg in the stomach to 3 mm Hg near the distal sigmoid colon, significantly exceeding those measured by electrodes.[16,18] One possible source of the discrepancy is that the charcoal probe was calibrated using aqueous suspensions, while the measurements were performed when the probe was in viscous fecal material. Large differences between the rates of oxygen diffusion in these 2 environments may have caused errors because the signal in EPR oximetry (EPR line width) is a function of both oxygen concentration and oxygen diffusion coefficient.

In the present study we adapted the phosphorescence quenching method[21] to measure oxygen levels in the intestinal lumen. By using a newly developed probe, we quantified oxygen levels in the intestinal lumen, gaining evidence that bacteria in the gut consume oxygen delivered by the colonic tissue. Furthermore, we show enrichment of oxygen-tolerant bacteria in the vicinity of the rectal mucosa in healthy human subjects as well as an unanticipated signature of asaccharolytic bacteria, which are dependent on the metabolism of proteinaceous substrates in the rectal mucus. Together, the compendium of bacteria showing aerotolerance and protein-based metabolism distinguish the mucosally associated microbiota from the feces. These bacterial taxa may play a role in the development of the dysbiotic microbiota associated with the inflammatory response in Crohn's disease and ulcerative colitis.[5]

Materials and Methods

Molecular oxygen quenches phosphorescence originating from excited triplet electronic states of molecules. The dependence of the phosphorescence lifetime (τ) on the Po_2 in the environment throughout the range of biological oxygen concentrations follows the Stern–Volmer model: $1/\tau = 1/\tau_0 + k_q \times Po_2$, where τ is the phosphorescence lifetime, and τ_0 and k_q are probe-specific parameters. By exciting an object containing a probe with a pulse of light and measuring the phosphorescence decay, Po_2 in the environment can be quantified. The measurements have a millisecond response, high specificity, and are independent of the probe distribution throughout the environment. The synthesis of the oxygen probe, the details of in vivo phosphorescence measurements, as well as the methods for acquisition of human samples, storage, and sequencing, can be found in the Supplementary Materials and Methods.

Animals

C57/B6 mice 8–12 weeks of age were used. All had free access to chow (AIN76; Research Diets, New Brunswick, NJ). Anesthesia was induced through a nose cone via inhalation of isoflurane 2.5% mixed with air, after which the isoflurane proportion was decreased to 1.5%. Low levels of isoflurane (<3%) do not cause significant changes in oxygenation.[22] Animals were anesthetized, and a laparotomy was performed in some cases (see the Discussion section for details). At the end of the experiments, the mice were euthanized according to the guidelines established by the American Veterinary Medical Association Panel on Euthanasia. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Results

Oxygen Measurements

The synthetic dendritic phosphorescent probes for tissue oxygen measurements[23–26] do not interact with proteins or other endogenous molecules, and the calibration parameters of these probes remain unchanged in any aqueous environment, ensuring absolute oxygen quantification.[27] In the present study, we used one such probe, Oxyphor G4,[24] to measure Po_2 in the intestinal tissue. The probe was injected into the tail vein in mice, and measurements were performed in reflectance-type geometry (Figure 1*A*). Excitation photons (λ_{ex} = 635 nm) are able to diffuse several centimeters into the tissue,[24,28] although excitation efficiency decreases exponentially with depth. The largest contribution to the signal is from the probe-containing layers closest to the fiber tip; however, in mice, the signal could be considered to be the average over the thickness of the entire intestinal wall, which is approximately 300-μm thick.

For quantitative oxygen measurements, the probe constants have to be determined in the same medium as encountered by the probe during measurements. In particular, quenching constant k_q depends on the rate of oxygen diffusion that differs between viscous intraluminal substances and water. Thus, calibration parameters obtained in solutions/suspensions are not suitable for measurements in the lumen.[19] Furthermore, properties of molecular probes, such as the Oxyphor G4, may be affected during their transit through the intestinal tract (eg, because of the actions of gut enzymes, bile acids, and/or interactions with processed food).

The quenching constant k_q is a function of the viscosity of the medium through which oxygen must diffuse during the probes' excited triplet lifetime (τ_0 = 200–300 μs for probes such as the Oxyphor G4). We reasoned that probe molecules dispersed in a large particle would predominantly encounter oxygen that traveled only within that particle. For example, in a 20-μm particle ($\sim 10^8$ probe molecules at a 50 μmol/L concentration), less than 1% of the probe will experience collisions with oxygen entering from the outside, assuming that the oxygen diffusion coefficient in the particle is 2×10^{-7} $cm^2 s^{-1}$ (ie, 100× lower than that in water, a typical value for solid polymers).[29,30] Hence, calibration of such a particle should be affected minimally by environmental properties, and oxygen equilibrium between the inside and the outside of the particle would be established in milliseconds.

Based on the above, a probe was prepared, OxyphorMicro, comprising polymethylmethacrylate (PMMA) particles