APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1995, p. 2681–2687 Vol. 61, No. 7
0099-2240/95/$04.00+0

The Termite Gut Microflora as an Oxygen Sink: Microelectrode Determination of Oxygen and pH Gradients in Guts of Lower and Higher Termites

ANDREAS BRUNE,[1]* DAVID EMERSON,[1,2] AND JOHN A. BREZNAK[1,2]

Department of Microbiology[1] and Center for Microbial Ecology,[2] Michigan State University, East Lansing, Michigan 48824-1101

Received 16 February 1995/Accepted 29 April 1995

Clark-type oxygen microelectrodes and glass pH microelectrodes, each with a tip diameter of ≤10 μm, were used to obtain high-resolution profiles of oxygen concentrations and pH values in isolated termite guts. Radial oxygen profiles showed that oxygen penetrated into the peripheral hindgut contents up to about 150 to 200 μm below the epithelial surface in both the lower termite *Reticulitermes flavipes* (Kollar) and the higher termite *Nasutitermes lujae* (Wasmann). Only the central portions (comprising less than 40% of the total volume) of the microbe-packed, enlarged hindgut compartments ("paunches") were completely anoxic, indicating that some members of the hindgut microbiota constitute a significant oxygen sink. From the slopes of the oxygen gradients, we estimated that the entire paunches (gut tissue plus resident microbiota) of *R. flavipes* and *N. lujae* accounted for 21 and 13%, respectively, of the respiratory activity of the intact animals. Axial oxygen profiles also confirmed that in general, only the paunches were anoxic in their centers, whereas midguts and posterior hindgut regions contained significant amounts of oxygen (up to about 50 and 30% air saturation, respectively). A remarkable exception to this was the posterior portion of an anterior segment (the P1 segment) of the hindgut of *N. lujae*, which was completely anoxic despite its small diameter (≈250 μm). Axial pH profiles of the guts of *Nasutitermes nigriceps* (Haldeman) and *Microcerotermes parvus* (Haviland) revealed that there were extreme shifts as we moved posteriorly from the midgut proper (pH ≈7) to the P1 segment of the hindgut (pH >10) and then to the P3 segment (paunch; pH ≈7). The latter transition occurred at the short enteric valve (P2 segment) and within a distance of less than 500 μm. In contrast, *R. flavipes*, which lacks a readily distinguishable P1 segment, did not possess a markedly alkaline region, and the pH around the midgut-hindgut junction was circumneutral. The oxic status of the peripheral hindgut lumen and its substantial oxygen consumption, together with previous reports of large numbers of aerobic and facultatively anaerobic bacteria in the hindgut microflora, challenge the notion that termite hindguts are a purely anoxic environment and, together with the steep axial pH gradients in higher termites, refine our concept of this tiny microbial habitat.

The presence of strictly anaerobic microorganisms in termite hindguts, especially the abundant cellulolytic flagellates in the lower termites, and the typical homoacetogenic and methanogenic processes and corresponding microbes involved in the dissimilation of carbohydrates in both lower and higher termites have led to the general concept that the termite hindgut is an anoxic habitat analogous to the rumen of cattle (reviewed in references 8 and 9). However, the results of studies on the composition of the cultivable bacterial flora (which have revealed the presence of strict aerobes), as well as estimates of the redox potentials in termite hindguts, have never been completely consistent with this concept. It has even been stated that termite hindguts are oxygen limited but basically aerobic (13), a conclusion reversed in a subsequent publication (25).

In part, the ambiguities surrounding the redox potential and/or oxygen status in termite guts arise from technical difficulties in making such measurements on minute amounts of material with little or no invasiveness or alteration of the native state. In part, the ambiguities are also semantic. Clearly, any statement about the physicochemical characteristics of an environment must be qualified with respect to the spatial resolving power of the measuring instrument or technique being used. The greater the spatial resolution, the more precise the description of the site in question. Terms such as "aerobic" or "microaerobic" really beg the question of the actual concentration of oxygen at the site, as well as the size and profile of the transition zone leading to anoxic conditions (i.e., the steepness of the oxygen gradient). If one is interested in the intestinal microecology of termite guts, which often have volumes on the order of 1 μl or less, then the closer that one can resolve site-to-site differences on the scale of individual microbial cells (i.e., in the micrometer range), the more refined the functional analysis of the microbial community and our interpretations of its potential impact on the host will be.

Previous efforts to characterize physicochemical conditions that exist in various regions of termite guts have made use of redox and pH indicator dyes, which were either fed to termites prior to dissection (13, 25) or added to contents extruded from dissected guts (4). pH measurements have also been made by using conventional combination electrodes and termite gut contents pooled in distilled water (4). Platinum electrodes have been used to measure the redox potentials in guts of two species of relatively large termites (*Zootermopsis nevadensis* and *Cubitermes severus*) (2). However, the dissected guts used for such determinations were covered with paraffin to retard the diffusion of oxygen to and through the gut tissue. This

* Corresponding author. Present address: Fakultät für Biologie, Mikrobielle Ökologie, Universität Konstanz, Postfach 5560 (M654), 78434 Konstanz, Germany. Phone: 49-7531-88 3282. Fax: 49-7531-88 2966. Electronic mail address: Andreas.Brune@uni-konstanz.de.

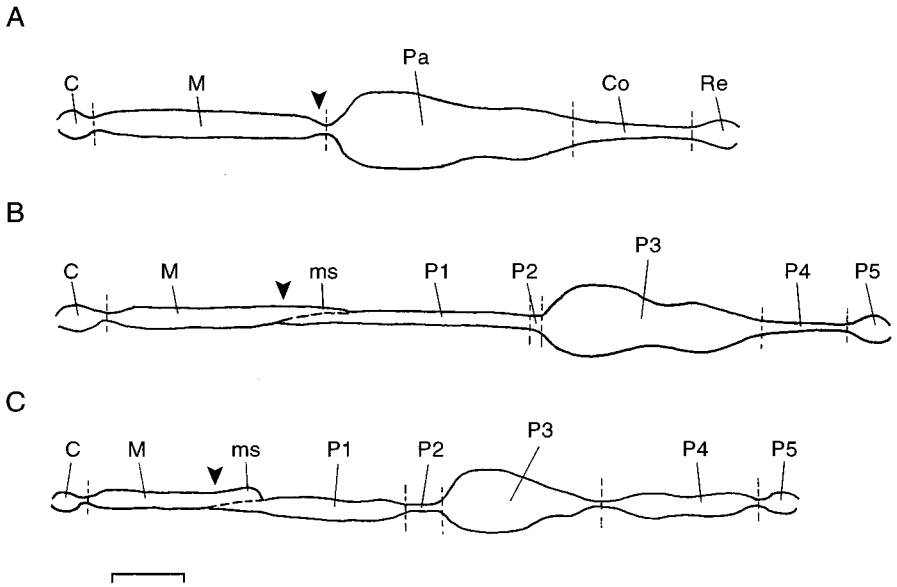

FIG. 1. Schematic diagram of the termite guts used in this study. (A) *R. flavipes*. (B) *Nasutitermes* species. (C) *M. parvus*. The gut of *R. flavipes* included the midgut (M), paunch (Pa), colon (Co), and rectum (Re). The hindguts of higher termites were differentiated into several proctodeal segments (P1 through P5); P3 is analogous to the microbe-packed paunch of *R. flavipes*, and P2 is the enteric valve. The mixed segment (ms) is a region in the gut of higher termites where both midgut and hindgut tissues are present. The crop (C) region of the foregut is also shown, but most guts tore just posterior to the crop during extraction from the termites. The arrowheads indicate the points of insertion of the Malpighian tubules. Bar = approximately 1 mm.

resulted in redox potentials which drifted toward negative values over periods of 5 to 15 min before some stability of the reading was achieved, and the reported redox potentials for the dilated, microbe-packed paunches (-47 mV for *C. severus* and -160 mV for *Z. nevadensis*) were means of individual values that spanned ranges of 110 and 230 mV, respectively. It must also be recognized that the redox potential is not a direct measure of oxygen concentration; relatively high (electropositive) redox potentials can occur in the complete absence of oxygen, and relatively low values are not necessarily inconsistent with an actively respiring yet oxygen-limited system. Thus, to evaluate the possible role of oxygen as a terminal electron acceptor or cosubstrate during metabolism of some of the termite gut microbes in situ, the oxygen concentration in the gut must be measured directly.

We sought to refine our understanding of the microenvironment in termite guts by exploiting the advances that have been made in microelectrode technology (23). This study was also prompted by results reported in the accompanying paper (11), which implicated oxygen as a critical cosubstrate for degradation of aromatic compounds by the termite gut microbiota. In this paper, we report in situ oxygen concentrations and pH values in various regions of the guts of higher and lower termites, as well as oxygen uptake rates for the microbe-packed paunches, which were measured on a microscale with electrodes having tip diameters of approximately 10 μm.

MATERIALS AND METHODS

Termites. *Nasutitermes lujae* (Wasmann) (Nasutitermitinae) and *Microcerotermes parvus* (Haviland) (Amitermitinae) were collected in the Mayombe rain forest in the Republic of Congo. *Nasutitermes nigriceps* (Haldeman), which was collected on the Cayman Islands, was a gift from M. Collins, Smithsonian Institution, Washington, D.C. These species, which are representatives of the so-called "higher termites" (family Termitidae), were kept in laboratory cultures by using a diet of white birch (*Betula papyrifera* Marsh.) and water. *Reticulitermes flavipes* (Kollar), representing one of the families of lower termites (Rhinotermitidae), was collected near Dansville, Mich., and was maintained in our laboratory as described previously (19). Worker larvae were used for all measurements.

The gut anatomies of the termites used in this study and the terms used for the various gut regions are shown in Fig. 1. The hindgut of *R. flavipes* consists of a single dilated paunch, a colon, and a rectum. *M. parvus* has the following three dilated proctodeal (hindgut) compartments besides the rectum (P5): P1, anterior to the enteric valve region (P2); P3, posterior to the enteric valve; and P4, analogous to the colon of *R. flavipes* and separated from P3 by a slightly narrowed zone (unlabeled). P1 and P4 of *Nasutitermes* species are not dilated significantly, and P2 is very short. The midgut-hindgut junction in all species is located at or near the insertion point of the Malpighian tubules. However, the higher termites also possess a so-called "mixed segment," just posterior to the Malpighian tubules, in which the intestinal lumen is limited on one side by a cuticulated hindgut wall and on the other side by an extension of the midgut epithelium. The term "paunch" is used in this paper to refer to the microbe-packed P3 region of the higher termites and the single hindgut dilation of *R. flavipes*. The terminology described above is consistent with that of Bignell and Anderson (4) and other authors.

Microelectrodes. Modified Clark-type oxygen microelectrodes containing guard cathodes (21) were constructed in our laboratory. These microelectrodes had tip diameters of 5 to 10 μm, 90% response times of <3 s, and stirring sensitivities of <2%. Prior to use, the electrodes were polarized overnight in deionized water that was continuously bubbled with air. They were calibrated by measuring the current when the microelectrode was placed in water saturated with air (21% O_2), as well as the background current in water sparged with 100% N_2 (0% O_2). Calibration was carried out before and after each experiment. The current was measured with a picoammeter (model 1201; Diamond-General, Ann Arbor, Mich.) connected to a strip chart recorder.

Glass pH microelectrodes were constructed in our laboratory by using a design described by Revsbech and Jørgensen (23). To minimize electrical noise, each microelectrode was shielded with an external casing containing 1 M KCl (16). The microelectrodes had tip diameters of 10 μm and response times of less than 30 s. They were calibrated with standard pH 4.0, 7.0, and 10.0 buffers before, during, and after each set of experiments. The average response was 53 mV per decade. The pH values were recorded with a high-impedance pH meter (Orion Instruments, Boston, Mass.) coupled to a chart recorder.

Measurements. For oxygen measurements, extracted termite guts (19) were embedded in agarose in the middle of glass microchambers which were 15 mm long, 10 mm high, and 2 or 4 mm thick. The microchambers were constructed from microscope slides and coverslips, both held vertically but separated from each other by three glass spacers (thickness, 2 or 4 mm), which formed the sides and bottom. A 1.5- to 4-mm agarose layer consisting of 0.5% agarose in insect Ringer's solution (7.5 g of NaCl per liter, 0.35 g of KCl per liter, and 0.21 g of $CaCl_2$ per liter) was cast into each microchamber. Then, a freshly extracted termite gut was placed flat and fully extended on this cushion and immediately

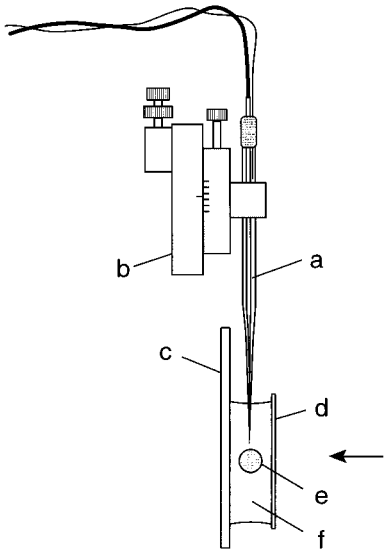

FIG. 2. Schematic side view of the experimental setup used for microelectrode measurements in termite guts: microelectrode (a) attached to micromanipulator (b) and microchamber back (c) and front (d) with gut (e) embedded in agarose (f). The angle of view with the dissecting microscope used is indicated by the arrow. The chamber was open to the air at the top and bottom; the glass sides of the chamber are not shown.

covered with an identical layer of molten agarose (40°C), which cooled and solidified immediately. After this, the glass spacer forming the microchamber bottom was removed, thus exposing the agarose-embedded gut to air both at the top and at the bottom of the microchamber in a symmetrical manner. The entire setup is shown diagrammatically in Fig. 2 and in operation in Fig. 3. For pH measurements, a similar setup was used, except that guts were placed on a 2-mm-thick bottom layer of 1.5% agarose and were covered with a 2-mm layer of 0.5% agarose.

The microelectrodes were positioned with a manual micromanipulator (Märzhäuser MM33; minimum step increment, 50 μm; Technical Products, Sarasota, Fla.). The progress of the tip was observed with a horizontally mounted stereomicroscope (Fig. 2). Usually, the fine microelectrode tip caused only a small deformation (<50 μm) of the gut wall before penetration. This could not be avoided, and since the deformed gut wall rebounded after it was punctured with the electrode, only negligible bias of the actual position of the electrode tip relative to the gut wall was created. All measurements were carried out at ambient temperature (22 ± 1°C).

Electron microscopy. Gut preparations used for transmission electron microscopy were fixed with glutaraldehyde, postfixed with OsO_4, and embedded in Epon. Thin sections were poststained with uranyl acetate and lead citrate. A detailed description of the method used has been published previously (10).

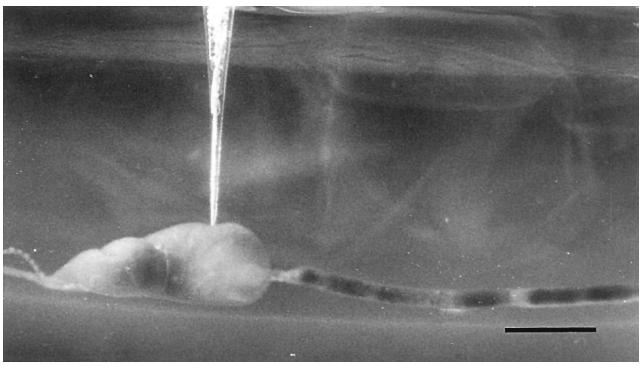

FIG. 3. Oxygen microelectrode in operation, inserted into the anterior P3 region of an *N. lujae* gut embedded in a microchamber. Note the black color of the alkaline P1 contents. Bar = 1 mm.

RESULTS

The termite guts usually separated at the foregut-midgut junction during extraction, yielding a nearly complete midgut attached to the hindgut. All of the guts retained peristalsis for 30 to 60 min while they were in the microchambers, indicating that the gut wall was physiologically active during the measurements. Moreover, puncturing the gut wall with the electrode triggered a brief increase in peristalsis. The establishment of steady-state oxygen gradients was verified by positioning the electrode tip halfway between the agarose surface and the gut and ascertaining that the oxygen signal was constant. Steady state was reached about 10 min after the gut was embedded in agarose and lasted for more than 1 h.

Radial oxygen profiles. Even with the very first measurement, it was obvious that the paunch region of termite hindguts was a significant oxygen sink. Figure 4A shows typical radial oxygen profiles for the paunch of *N. lujae*. Similar profiles were obtained for the paunch of *R. flavipes* (Fig. 4C). In all cases, there was a linear initial decrease in O_2 concentration from the ambient concentration at the agarose surface toward the gut. However, the steepness of the gradient increased and became curvilinear as the electrode tip approached the gut wall. Once the electrode tip penetrated the gut wall, the oxygen concentration decreased even more rapidly, but oxygen was detectable until the tip was about 150 to 200 μm below the exterior surface of the gut wall, so that only the central region of the gut lumen was anoxic. Inasmuch as the gut walls of the termites, including the enveloping muscle fibers, were only about 5 to 20 μm thick (Fig. 5), our findings implied that there was a "shell" of oxygen-containing gut fluid up to almost 200 μm inward from the apical surface of the paunch epithelium. Continued advancement of the microelectrode tip through the gut until it emerged on the opposite side yielded an oxygen profile that was the mirror image of that obtained during entry of the electrode into the gut. This indicated that the oxygen present in the ca. 200-μm region below the exterior gut surface was not due to an intrusion of oxygen accompanying the puncture of the gut with the microelectrode tip, but was due to actual diffusion of oxygen through the gut wall.

Embedding guts in agarose restricted the diffusion of oxygen to them. Hence, the depth at which a gut was embedded influenced the absolute oxygen concentration at its surface and the depth of penetration of oxygen into it. However, we chose to embed guts in agarose for two major reasons: (i) to prevent the otherwise rapid desiccation of the guts during measurements; and (ii) to facilitate the measurement of oxygen fluxes into the guts caused by the consumption of oxygen in them. Thus, although the initial slope of the oxygen gradient above any specific portion of a gut (i.e., within the agarose layer) decreased when the depth at which it was embedded in the agarose was increased, the slope was quite reproducible at a given depth. For example, the initial slopes of the oxygen gradients were 101 ± 16 μM mm^{-1} (n = 7) above the median region of the paunch (P3) of *N. lujae* and 141 ± 17 μM mm^{-1} (n = 5) above the median region of the paunch of *R. flavipes* when each was embedded ca. 1.5 mm below the agarose surface.

In all cases, the paunch was the most significant oxygen sink. The relatively narrow, tubular midguts of all species never exhibited as significant an oxygen gradient around them as the paunches did, and they also never became anoxic (Fig. 4B and C). However, the posterior portion of the first proctodeal segment (P1) of *N. lujae*, which had roughly the same outside diameter as the midgut, exhibited a small but significant oxygen gradient around it, and once the gut wall of this portion was

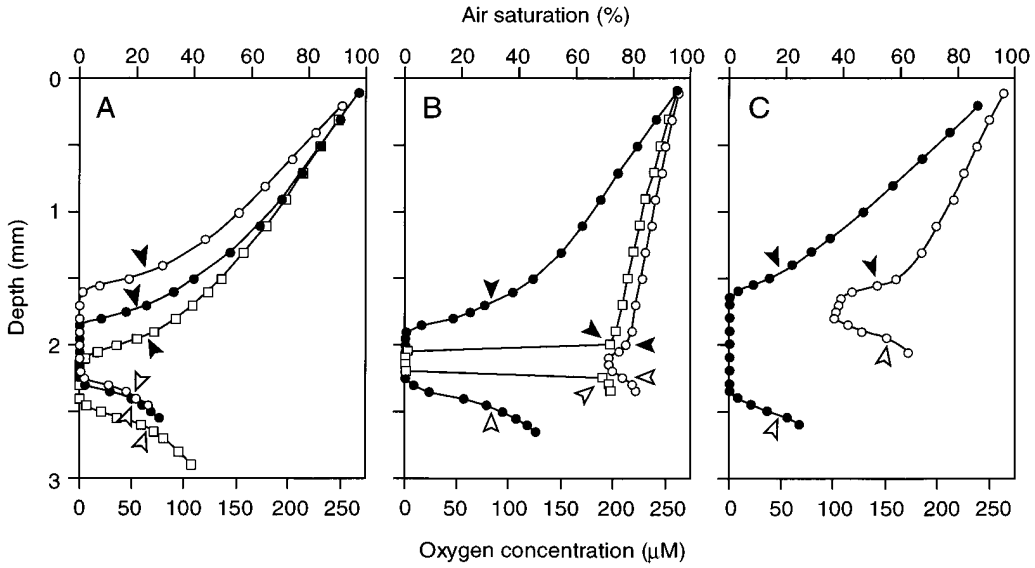

FIG. 4. Radial oxygen profiles of termite guts. Depth refers to the distance between the electrode tip and the surface of the agarose. To account for individual variations in the exact depths of embedding, the solid arrowheads indicate the points at which the tip reached the gut wall; the open arrowheads indicate the point of emergence on the opposite side. (A) Profiles obtained at the anterior (●), median (○), and posterior (□) P3 region of a typical *N. lujae* hindgut. (B) Profiles obtained in a separate experiment with another *N. lujae* gut, illustrating the difference between the median midgut (○), posterior P1 (□), and anterior P3 (●) regions. (C) Profiles for the median midgut (○) and paunch (●) of a typical *R. flavipes* preparation. The thicknesses of the microchambers used were 4 mm for *N. lujae* and 2 mm for *R. flavipes*.

penetrated, the oxygen concentration decreased to zero within 50 μm (Fig. 4B).

Axial oxygen profiles. The anterior-to-posterior oxygen profiles of termite guts (i.e., the axial oxygen profiles), determined by measuring the concentration in the center of each gut region, are shown in Fig. 6. Because of the artificial restriction of the oxygen flux toward the gut that resulted from being embedded in agarose, the absolute oxygen concentrations measured in the guts must be interpreted with caution. In vivo, the gut epithelium, which is intimately tracheated, may well have a better oxygen source than the oxygen supply provided in the microchambers, but no absolute values are known. Thus, the axial oxygen profiles shown in Fig. 6 probably represent conservative estimates of the oxygen concentrations in the guts of *N. lujae* and *R. flavipes*. Nevertheless, these profiles illustrate the relative oxygen status in the various gut regions. In both termites, the center of the paunch region of the hindgut was always anoxic. The midguts had oxygen concentrations that were only slightly lower than the oxygen concentrations outside the guts. As mentioned above, however, the narrow P1 region of *N. lujae* exhibited a rapid decrease in oxygen partial pressure along its axis and was completely anoxic in its posterior region.

Axial pH profiles. Figure 7 shows typical axial pH profiles around the midgut-hindgut junction for three different termite species, which were determined by measuring the pH in the center of each gut region. Whereas both the midgut and the paunch had pH values around 7 in both lower and higher termites (pH 6.68 ± 0.17 and 6.38 ± 0.19 for *R. flavipes*, pH 6.82 ± 0.24 and 6.78 ± 0.38 for *N. nigriceps*, and pH 6.99 ± 0.37 and 7.39 ± 0.32 for *M. parvus* for the median midgut and anterior paunch, respectively; $n = 5$), the P1 segments of the higher termites became increasingly alkaline along their axes, reaching pH values of more than 10 in their posterior portions (pH 10.23 ± 0.46 for *N. nigriceps* and pH 10.66 ± 0.32 for *M. parvus*; $n = 5$) (Fig. 7A and B). Across the enteric valve (P2) and into the anterior P3 region, however, the pH decreased sharply more than 3 pH units within 0.5 mm. The midgut-hindgut region of *R. flavipes* had no markedly alkaline portion; in this termite the transition from posterior midgut to anterior paunch was accompanied by a decrease in pH of less than 1 pH unit (Fig. 7C).

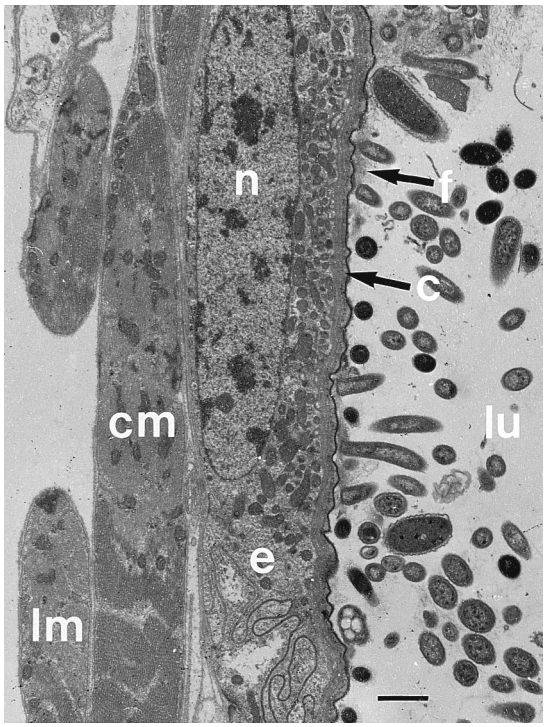

FIG. 5. Transmission electron micrograph of a portion of the paunch wall of *R. flavipes*. The epithelium (e) consists of a single cell layer and is based on an equally thin layer of circular (cm) and longitudinal (lm) muscle fibers. Note the numerous mitochondria next to the nucleus (n). The cuticular layer (c) of the epithelium is very thin (<0.25 μm) and bears numerous bacteria on its luminal side (lu), some of which are attached by fibrous holdfast material (f). Bar = 1 μm.

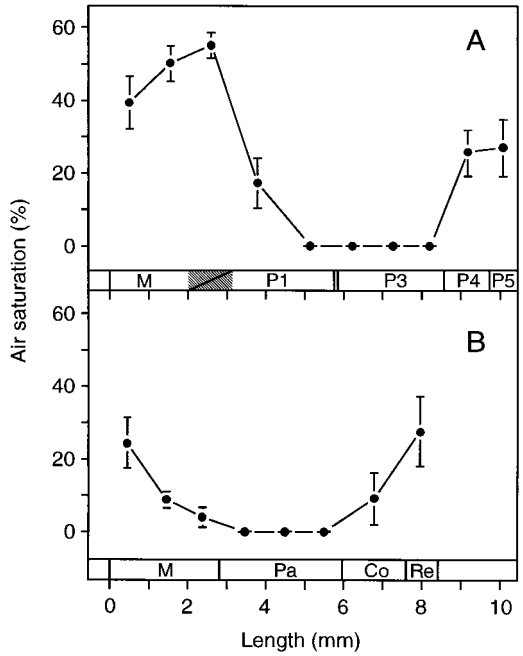

FIG. 6. Axial average O_2 concentration profiles in guts of *N. lujae* (A) and *R. flavipes* (B). Values were determined at the center of each gut region; the bars indicate standard deviations ($n = 5$). The shaded area indicates the position of the mixed segment in *N. lujae*. See the legend to Fig. 1 for explanations of the abbreviations used for gut regions.

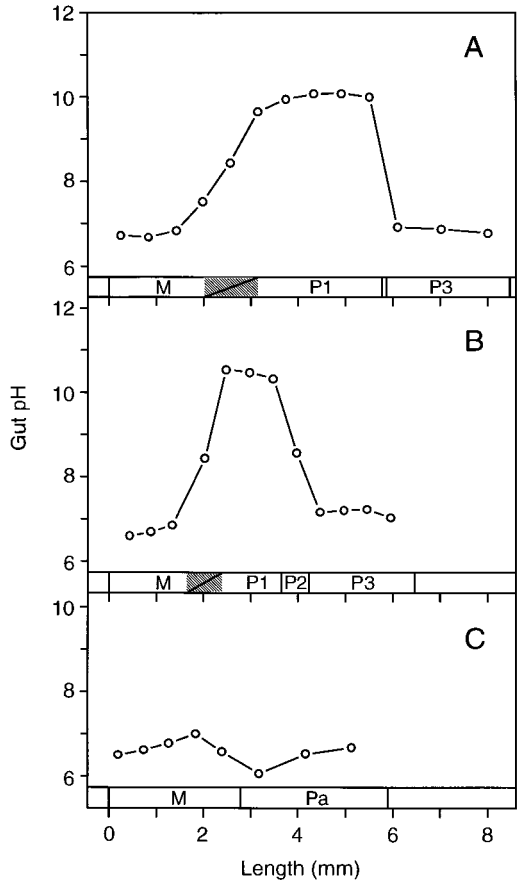

FIG. 7. Typical axial pH profiles in the midgut-hindgut regions of *M. parvus*. (A), *N. nigriceps* (B), and *R. flavipes* (C). Values were determined at the center of each gut region. The shaded areas indicate the positions of the mixed segments in the higher termites. See the legend to Fig. 1 for explanations of the abbreviations.

DISCUSSION

The results of our fine-scale resolution of the oxygen and pH gradients that exist in termite guts illustrate the futility of trying to describe this habitat by using general terms such as "oxic" and "anoxic" or "acidic" and "alkaline." In fact, the data force us to refine our concept of the gut as being quite structured, not only anatomically but with regard to microenvironments that are present in the gut, with distinct and sometimes remarkably abrupt zones of transition from one physicochemical state to another. Although an understanding of microenvironmental quality is critical to a functional analysis of any microbial community, it becomes all the more important when the size of the habitat is small. Such is clearly the case for termite guts, whose dimensions are often measured in fractions of a millimeter or microliter.

Crude measurements obtained with live termites indicated that anoxic conditions were present after the electrode tip was forced through the hard integument into the abdomen (data not shown), but spatially defined measurements were impossible since the electrode always penetrated deeply and uncontrollably into the paunch. Embedding guts in agarose allowed us to overcome this problem, and we were not surprised to find a significant amount of oxygen present near the apical surface of the paunch epithelium (e.g., 50 to 100 μM in *N. lujae* and *R. flavipes*) (Fig. 4), as this is a transporting epithelium whose uptake of microbial fermentation products (e.g., acetate) is important to termite nutrition and whose apical region is rich in mitochondria (Fig. 5) (10). However, we were struck by the relatively large zone occupied by the diminishing oxygen gradient (100 to 200 μm inward from the epithelial surface) before total anoxia occurred in the central portion of the paunch. By approximating the paunch to a cylinder, whose axis lies at the center of the anoxic zone, we calculated from Fig. 4 that only 25 to 40% of the entire paunch volume of our gut preparations was completely anoxic under experimental conditions. This is consistent with the presence of strictly anaerobic bacteria and protozoa in termite guts, as well as the presence of typical fermentation products of anaerobic microbial metabolism on which termites thrive (8, 9). It also explains the presence of strictly aerobic and facultatively anaerobic bacteria as numerically significant members of the gut flora of both lower and higher termites (6, 14, 24). It does not tell us, however, whether the same O_2 gradient profiles exist in situ (see below), whether strict anaerobes are capable of avoiding all contact with oxygen, or how oxygen-consuming organisms might capitalize on the presence of oxygen. Electron microscopy has revealed that the hindgut epithelium is colonized by a diverse array of prokaryotes, which often possess, or are embedded in, some sort of extracellular holdfast material (Fig. 5) (10, 12). It might be expected that some members of this attached flora are oxygen consumers and benefit by being in the peripheral, oxic zone. Likewise, prokaryotes attached to cuticular spines protruding into the gut lumen of certain termites (5) may be strict anaerobes. However, there are no data to confirm these ideas yet. In truth, we are still largely ignorant of the in situ distribution of specific microbial taxa in termite guts and the extent to which specific metabolic types are exposed to varying microenvironmental conditions as a result of mixing or cell motility.

What is clear from our results, however, is that some mem-

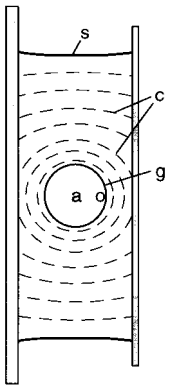

FIG. 8. Schematic representation of the oxygen concentration gradient around the termite hindgut at steady state in the experimental setup shown in Fig. 2. The concentration isolines (c) illustrate that the initial slope of the oxygen profile below the agarose surface (s) is quasilinear because of the narrow geometry of the microchamber. The shaded area (a) indicates the anoxic center of the gut lumen, which is surrounded by the microoxic periphery (o). The solid line (g) represents the outer surface of the gut wall; isolines within the gut were omitted for clarity. See Discussion for details.

bers of the termite gut microbiota constitute a significant oxygen sink and help create anoxic conditions in the central region of the paunch. This was suggested more than a decade ago on the basis of crude estimates of the redox potentials in guts before and after depression of the bacterial population with antibiotics (26). Now, with the aid of microelectrodes, not only were we able to make direct microscale measurements of the oxygen flux to and through the guts, but we could also estimate the contribution that the paunch and its microbiota might make to the total respiratory rate of the intact animals. By using narrow microchambers and guts embedded in agarose, it was possible to model oxygen consumption as a one-dimensional system, as illustrated in Fig. 8. The slope of the oxygen profile could then be used to calculate the oxygen uptake by the gut by using the one-dimensional version of Fick's first law of diffusion: $J = -\phi D_s \, \delta C(x)/\delta x$ (23), where J is the flux of molecules through a unit of area per unit of time, ϕ is the porosity, D_s is the diffusion coefficient, and $C(x)$ is the concentration of molecules at depth x. For porosity and the diffusion coefficient of oxygen in agarose gels we used the values for pure water ($\phi = 1$; $D_s = 2.1 \times 10^{-9}$ m^2 s^{-1} at 22°C), which were found to be applicable at agar concentrations up to 2% and over a wide range of salinity values (22). Using the average concentration gradients of oxygen above paunches of *N. lujae* (the P3 region) and *R. flavipes* (101 and 141 μM mm^{-1}, respectively) (see Results), we calculated fluxes of 212 and 296 nmol m^{-2} s^{-1}, respectively. To estimate the total oxygen consumption in the P3 region of *N. lujae* and the paunch of *R. flavipes*, we assumed that the area (A) of the agarose surface above this gut region was $A = 2\,l\,w$, where l is the length of the paunch if we assumed that it approximated a cylinder (2.0 and 2.5 mm for *N. lujae* and *R. flavipes*, respectively) and w is the thickness of the microchamber (4 and 2 mm, respectively). We then multiplied by 2 to account for oxygen diffusion through the top and bottom surfaces of the symmetrical chamber. Thus, the oxygen uptake in that gut region (R_G) was $R_G = JA$ and was calculated to be 3.39 pmol s^{-1} for *N. lujae* and 2.96 pmol s^{-1} for *R. flavipes*. Compared with the total respiratory activity of the termites, which was assumed to be 26 pmol of O_2 s^{-1} for *N. lujae* (calculated by using a specific oxygen consumption value of 1 μl of O_2 mg of termite^{-1} h^{-1} [20] and a fresh weight of 2.1 mg per larva), and was determined previously to be 14.3 pmol of O_2 s^{-1} for laboratory-maintained worker larvae of *R. flavipes* (19), the oxygen consumption in the P3 region or paunch could account for roughly 13 and 21% of the total respiratory activity of *N. lujae* and *R. flavipes*, respectively. These estimates must be viewed with caution, however, because the normal supply of oxygen to the gut wall and hence the size and shape of the oxygen gradient in the gut in situ are not known. Inasmuch as such transport would be mainly via tracheal diffusion in a gaseous phase, embedding the guts in agarose may have significantly limited diffusion, resulting in measured flux values that were lower than those that occur in situ. On the other hand, intact termites may themselves limit, by spiracular closure or other mechanisms (18), the tracheal oxygen supply to the gut in order to promote the formation of microbial fermentation (i.e., incomplete oxidation) products on which they thrive. Such limitation was not possible in our case, in which the isolated guts may have received a greater oxygen supply than they would have in situ. In any case, although the epithelial cells and muscular layers of the gut wall contribute to oxygen consumption and must be considered an oxygen sink even under experimental conditions, the oxygen gradients shown in Fig. 4 reveal that there was only a minor decrease in the oxygen concentration across the relatively thin gut wall. The major oxygen sink clearly was in the peripheral region of the gut lumen and must be attributed to some members of the resident microbiota. If the viability of the peripheral hindgut microbiota was affected by brief exposure to elevated temperatures during the embedding procedure (although this is unlikely in view of the retention of gut peristalsis throughout the experiment), then the in situ oxygen uptake rates would have been even higher and would have been accompanied by decreased oxygen penetration into the gut periphery.

Whereas anoxic conditions in the centers of the paunches can be explained by the relatively large paunch diameters and the apparent respiratory activity of microorganisms near the epithelium, the anoxic conditions in the posterior P1 region of *N. lujae* are more difficult to explain (Fig. 4B). Despite the small diameter of this region (≈250 μm), the oxygen concentration plummeted to zero almost immediately below the gut wall. At present, we do not know whether this was attributable to a high respiration rate of the epithelial tissue and/or the gut microbes in this region or whether the chitinous lining of the epithelium in the P1 region represents a greater diffusion barrier to oxygen than it does in the P3 region.

pH microelectrodes allowed us to confirm, in general and with significantly improved spatial resolution, measurements made with termites by other workers and to expand the roster of tested species. The results which we obtained with *R. flavipes* were typical of those obtained with lower termites in general (4) in that the midgut-hindgut contents were circumneutral and there was a small yet significant decrease of 1 pH unit at the midgut-hindgut junction. In contrast, the P1 regions of the hindguts of the higher termites were quite alkaline, reaching pH values greater than 10 (Fig. 7); these are among the highest values reported for any termites (3, 4), but similarly high values have been found in mid- or hindguts of other insects (1, 15, 17). Our results obtained with wood-feeding higher termites show that the increase in pH begins just posterior to the insertion point of the Malpighian tubules and continues along the mixed segment and that the highest pH values occur in the P1 region. This is in agreement with the proposal of Bignell and coworkers, who suggested that alkaline conditions in the P1 and P3 regions of soil-feeding higher termites are related to the increased potassium ion concentrations probably secreted by the mesenteral lining of the mixed segment (7). What is remark-

able, however, is the abruptness with which the pH returned to neutral in the wood-feeding species which we studied. This transition occurred along the P2 region, which comprises the enteric valve and which in *N. nigriceps* is only about 200 μm long (Fig. 7B). Marked alkalinity in the anterior hindgut region seems to be an evolutionary trend among the higher termites. The physiological significance of such alkalinity may lie in some sort of chemical pretreatment (e.g., alkaline peroxidative depolymerization) of polyaromatic plant material (lignin, humus) before it is passed down the gut for further degradation, and it might be related to the rapid oxygen consumption in the P1 region and the dark color of the P1 contents observed frequently in *Nasutitermes* guts (Fig. 3), but so far there is no experimental evidence for this.

The results of our fine-scale resolution of pH and oxygen gradients in termite guts helped us refine our concept of the microenvironments that occur in this tiny habitat. They also help explain the dependence of the termite gut microbiota on oxygen for the degradation of aromatic compounds, as reported in the accompanying paper (11). It may well be that aromatic compound degraders are one component of the microflora that constitutes an oxygen sink in termite guts. We hope that our results will encourage further studies to clarify the importance of oxygen-consuming and alkaliphilic microbes in the digestion of plant material in termite guts.

ACKNOWLEDGMENTS

This work was funded by National Science Foundation grant IBN9106636 to J.A.B. and National Science Foundation grant BIR9120006 to the Center for Microbial Ecology. A.B. was supported by a Feodor-Lynen fellowship from the Alexander von Humboldt Foundation, Germany.

We are grateful to H. S. Pankratz for help with electron microscopy and to M. Collins (Smithsonian Institution, Washington, D.C.) for providing specimens of *N. nigriceps*. We also thank J. R. Leadbetter for helpful discussions.

REFERENCES

1. **Bayon, C.** 1980. Volatile fatty acids and methane production in relation to anaerobic carbohydrate fermentation in *Oryctes nasicornis* larvae (Coleoptera: Scarabaeidae). J. Insect Physiol. **26:**819–828.
2. **Bignell, D. E.** 1984. Direct potentiometric determination of redox potentials of the gut contents in the termites *Zootermopsis nevadensis* and *Cubitermes severus* and in three other arthropods. J. Insect Physiol. **30:**169–174.
3. **Bignell, D. E.** 1994. Soil-feeding and gut morphology in higher termites, p. 131–158. *In* J. H. Hunt and C. A. Nalepa (ed.), Nourishment and evolution in insect societies. Westview Press, San Francisco.
4. **Bignell, D. E., and J. M. Anderson.** 1980. Determination of pH and oxygen status in the guts of lower and higher termites. J. Insect Physiol. **26:**183–188.
5. **Bignell, D. E., H. Oskarsson, and J. M. Anderson.** 1979. Association of actinomycete-like bacteria with soil-feeding termites (Termitidae, Termitinae). Appl. Environ. Microbiol. **37:**339–342.
6. **Bignell, D. E., H. Oskarsson, and J. M. Anderson.** 1980. Distribution and abundance of bacteria in the gut of a soil-feeding termite *Procubitermes aburiensis* (Termitidae, Termitinae). J. Gen. Microbiol. **117:**393–403.
7. **Bignell, D. E., H. Oskarsson, J. M. Anderson, and P. Ineson.** 1983. Structure, microbial associations and function of the so-called "mixed segment" of the gut in two soil-feeding termites, *Procubitermes aburiensis* and *Cubitermes severus* (Termitidae, Termitinae). J. Zool. (London) **201:**445–480.
8. **Breznak, J. A.** 1982. Intestinal microbiota of termites and other xylophagous insects. Annu. Rev. Microbiol. **36:**323–343.
9. **Breznak, J. A., and A. Brune.** 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu. Rev. Entomol. **39:**453–487.
10. **Breznak, J. A., and H. S. Pankratz.** 1977. In situ morphology of the gut microbiota of wood-eating termites [*Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki]. Appl. Environ. Microbiol. **33:**406–426.
11. **Brune, A., E. Miambi, and J. A. Breznak.** 1995. Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. Appl. Environ. Microbiol. **61:**2688–2695.
12. **Czolij, R., M. Slaytor, and R. W. O'Brien.** 1985. Bacterial flora of the mixed segment and the hindgut of the higher termite *Nasutitermes exitiosus* Hill (Termitidae, Nasutitermitinae). Appl. Environ. Microbiol. **49:**1226–1236.
13. **Eutick, M. L., R. W. O'Brien, and M. Slaytor.** 1976. Aerobic state of gut of *Nasutitermes exitiosus* and *Coptotermes lacteus*, high and low caste termites. J. Insect Physiol. **22:**1377–1380.
14. **Eutick, M. L., R. W. O'Brien, and M. Slaytor.** 1978. Bacteria from the gut of Australian termites. Appl. Environ. Microbiol. **35:**823–828.
15. **Felton, G. W., and S. S. Duffey.** 1991. Reassessment of the role of gut alkalinity and detergency in insect herbivory. J. Chem. Ecol. **17:**1821–1836.
16. **Jensen, K., N. P. Revsbech, and L. P. Nielsen.** 1993. Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate. Appl. Environ. Microbiol. **59:**3287–3296.
17. **Martin, M. M., J. S. Martin, J. J. Kukor, and R. W. Merritt.** 1980. The digestion of protein and carbohydrate by the stream detritivore, *Tipula abdominalis*. Oecologia (Berlin) **46:**360–364.
18. **Nation, J. L.** 1985. Respiratory systems, p. 185–225. *In* M. S. Blum (ed.), Fundamentals of insect physiology. John Wiley & Sons, New York.
19. **Odelson, D. A., and J. A. Breznak.** 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl. Environ. Microbiol. **45:** 1602–1613.
20. **Peakin, G. J., and G. Josens.** 1978. Respiration and energy flow, p. 111–163. *In* M. V. Brian (ed.), Production ecology of ants and termites. Cambridge University Press, Cambridge.
21. **Revsbech, N. P.** 1989. An oxygen microelectrode with a guard cathode. Limnol. Oceanogr. **34:**472–476.
22. **Revsbech, N. P.** 1989. Diffusion characteristics of microbial communities determined by use of oxygen microsensors. J. Microbiol. Methods **9:**111–122.
23. **Revsbech, N. P., and B. B. Jørgensen.** 1986. Microelectrodes: their use in microbial ecology, p. 293–352. *In* K. C. Marshall (ed.), Advances in microbial ecology. Plenum, New York.
24. **Schultz, J. E., and J. A. Breznak.** 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Appl. Environ. Microbiol. **35:**930–936.
25. **Veivers, P. C., R. W. O'Brien, and M. Slaytor.** 1980. The redox state of the gut of termites. J. Insect Physiol. **26:**75–77.
26. **Veivers, P. C., R. W. O'Brien, and M. Slaytor.** 1982. Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. J. Insect Physiol. **28:**947–951.